1997

Replication of barley yellow dwarf luteovirus-PAV RNA

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Replication of barley yellow dwarf luteovirus-PAV RNA

by

Mohan Bangalore

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Molecular, Cellular, and Developmental Biology

Major Professor: W. Allen Miller

Iowa State University

Ames, Iowa

1997

Graduate College

Iowa State University

This is to certify that the doctoral dissertation of

Mohan Bangalore

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program

Signature was redacted for privacy.

For the Graduate College

This dissertation is dedicated to my parents

SHANTHA and **RAMASWAMY**

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I am grateful to Dr. W. Allen Miller, my major professor, for his constant support, help and co-operation during the course of my Ph.D. study.

I am thankful to my program of study committee members: Dr. John Hill, Dr. McNabb, Dr. James Colbert and Dr. Steven Rodermel for valuable discussion and helpful advice. I sincerely acknowledge the support of my collaborators: Dr. S. P. Dinesh-Kumar, Dr. Silver, Dr. David Somers and G. Koev.

I thank my colleagues and friends: Dr. Shanping Wang, Dr. Lada Rasachova, Dr. Cindy Paul, Dr. Micheal Walter, Dr. Chris Brown, Dr. Eggenberger, Randy Beckett, Michele Aulik, G. Koev, Guo Liang, Bill Hites, Raju Kakani, Harikrishnan and Taroon Kumar for thier useful suggestions and stimulating discussions.

I am extremely grateful to my parents (Shantha and Late. Ramaswamy), granny (Byamma), brothers (Srinivas and Muralidhar) and sister-in-law (Poornima) for their endless support and patience. I am especially thankful to my wife Arathi, for an extraordinary amount of help, inspiration and co-operation. I extend my gratitude to my uncles (Kitti and Nagesha), aunts (Latha and Nagalakshmi), cousins (Chandru, Venka and Bharathi), in-laws (Nagaraja Iyer, Ajji, Preethi, Ananthu, Jyothi and Praveen Murthy) and all my friends and family for their support and encouragement.

ABSTRACT

Barley yellow dwarf luteovirus (BYDV)-PAV serotype, an economically important virus of small grain cereals, has a positive-sense RNA genome encoding at least six open reading frames (ORFs). The goal of the research was to determine the genes and sequences involved in the viral replication and to design efficient antiviral strategies to BYDV-PAV. Genetically engineered resistance is essential for BYDV as the natural resistance is inadequate. Antiviral constructs such as sense RNA, antisense RNA and viral polymerase gene were tested for their ability to reduce virus titre in oat protoplasts as monitored by enzyme-linked immunosorbent assay. All antiviral constructs yielded low levels of viral antigen. However, none of the above constructs showed decrease in viral RNA accumulation in Northern blot analysis. Deletion and mutation analyses were performed to determine genes and *cis*-acting signals involved in translation, replication and encapsidation of BYDV-PAV. ORFs 1 and 2, which encode the putative polymerase gene, were required for replication. Deletion of the coat protein (CP) gene reduced the accumulation of genomic RNA. The carboxy-terminally extended form of the CP was not necessary for replication or encapsidation. *Cis*-acting RNA signals in and around ORF6 were essential for viral replication. BYDV-PAV replication may be coupled to translation because defective RNAs containing various deletions were not replicated in *trans* by the co-inoculated wild-type helper genome. Site-directed mutagenesis was used to map the subgenomic RNA1 (sgRNA1) promoter because subgenomic promoters are putative hotspots of viral recombination and putative replication origin. Mutating the sgRNA1 transcription initiation base, G at 2670, or the nucleotides immediately flanking it, reduced

sgRNA1 accumulation. Computer-predicted secondary structures in the putative sgRNA1 promoter regions of many members of subgroup I luteovirus has revealed a conserved stem-loop structure near the sgRNA1 start site. Mutating a conserved ACAAA motif reduced both the genomic RNA and sgRNA1 accumulation. A premature stop codon introduced at base 2650, 90 bases from the 3' end of the polymerase gene, abolished BYDV-PAV replication in oat protoplasts.

CHAPTER 1. GENERAL INTRODUCTION

Luteoviruses

Barley yellow dwarf virus (BYDV) is the type member of luteovirus group of plant viruses. Luteoviruses are spherical, phloem-limited, obligately aphid-transmitted plant viruses. Even though the effects of luteovirus infection has long been known in the class of "yellowing" diseases (Duffus, 1977), luteoviruses were first recognized as a group of related viruses by Shepherd *et al.* (1976). The characteristic yellowing (hence the prefix luteo- from Latin *luteus,* yellow) or reddening of the foliage and rolling of leaves reflect the pathological effects of infection on the phloem tissue of the host. The virus particle consists of 180 subunits of the 22-kDa coat protein (CP), arranged as an icosahedron with a diameter of 24 to 30 nm. A few copies of the CP may contain an additional 50- to 53-kDa polypeptide fused to carboxy-terminus of the CP, produced by translational readthrough of the CP stop codon (Brown *et al.*, 1996). The thermal inactivation points for luteoviruses range from 50 to 65°C (Rochow, 1970).

Based on serological relationships, cytopathological effects, symptoms, host range, aphid vector specificity and genome organization, luteoviruses are classified into two subgroups (D'Arcy, 1986). Subgroup I of luteoviruses includes the PAV, MAV and SGV serotypes of BYDV, soybean dwarf virus (SDV) and others. Subgroup II includes the RPV serotype of BYDV, beet western yellows virus (BWYV), potato leafroll virus (PLRV) and others.

Luteoviruses are the most widespread and economically important viruses of potatoes, sugar beets and small grain cereals such as barley, wheat and oats, worldwide (Rochow and Duffus, 1981; D'Arcy and Burnett, 1995). Most luteoviruses have limited host ranges. For example, plant hosts of BYDV and SDV come from only one family, Graminae and Leguminosae, respectively (Mayo and Zeigler-Graff, 1996). However, BWYV infects plant species of 23 dicotyledonous families (Rochow and Duffus, 1981).

The characteristic symptoms of luteoviral infection include stunting of infected plants, yellowing, reddening, rolling, and brittleness of infected leaves (Rochow and Duffus, 1981). Symptoms vary with the plant species, crop variety and other factors. By increasing the number of sterile florets and by reducing the kernel weight, BYDV can cause significant yield losses in crop plants. Luteoviruses are confined to the phloem, mostly found in sieve elements, phloem parenchyma and companion cells (Rochow, 1969; Waterhouse *et al.*, 1988). However, efficient replication of luteoviruses in protoplasts derived from mesophyll tissues or suspension cells has been demonstrated (Barker and Harrison, 1982; Young *et al.*, 1989; Dinesh-Kumar *et al.*, 1992; Veidt *et al.*, 1992).

Cytopathology

Subgroups I and II luteoviruses induce different cytopathological events in infected cells (Gill and Chong, 1979). In cells infected with subgroup I luteovirus; extreme distortion of nucleus, accumulation of densely staining, heterochromatin-like material and accumulation of new virus particles in the cytoplasm occurs. Cells infected

by subgroup II isolates show relatively normal nuclei at first, until the heterochromatin slowly disintegrates and the accumulation of new virus particles around the nucleolus. The cytopathological events induced by BWYV (Esau and Hoefert, 1972) and PLRV (Shepardson *et al.*, 1980) are similar to those of subgroup II stains of BYDV.

Virus transmission

Luteoviruses are transmitted to plants only by aphid vectors in a circulative, persistent manner (Rochow, 1969). Highly specialized virus-vector (aphid) relationship has allowed differentiation of BYDV isolates based on their predominant aphid vector. Names of serotypes of BYDV are acronyms derived from the names of aphid species that transmit them. For example, MAV is specifically transmitted by *Sitobion* (formerly *Macrosiphum*) *avenae*, SGV by *Schizaphis graminum* and RPV by *Rhopalosiphum padi*. PAV is transmitted by both *Rhopalosiphum padi* and *Sitobion avenae*. Virus particles ingested through the stylet, accumulate in hindgut, from which it is transported across the gut lining into the hemocoel. From hemocoel, the virus particles are transported to the basal membrane of the accessory salivary gland, from which it is exuded into the salivary duct. Virions are then excreted along with the salivary cell secretions into the phloem cells of healthy plants during feeding (Gildow and Rochow, 1980; Gildow 1982). The particles appear to cross both the hindgut cells and the accessory salivary gland membranes via coated vesicles (Gildow, 1993; Gildow and Gray, 1993). Many factors such as length of

acquisition, inoculation access period, virus concentration, and others have been shown to affect the efficiency by which aphids transmit the virus (Power and Gray, 1995).

Genome organization and gene functions

The genome of a luteovirus consists of a positive-sense RNA that is 5.5 to 5.7kb long and encodes up to six open reading frames (ORFs; for reviews see Miller, 1994; Miller *et al.*, 1995; Mayo and Zeigler-Graff, 1996). A 5'-terminal genome-linked protein (VPg) has been reported for PLRV (Mayo *et al.*, 1982) and BYDV-RPV (Murphy et al., 1989). BYDV-PAV lacks a Vpg (E. Allen, personal communication). The 3' end of the genome does not contain a poly (A) tail or a tRNA-like structure.

The genome organization of subgroup I and II luteoviruses is shown in Figure 1. Subgroup I luteoviruses contain an ORF encoding a 4.3-6.7-kDa protein (ORF6) which is absent in subgroup II viruses. Subgroup II luteoviruses have an ORF at the 5' end of the genome (ORF0) that encodes a 28-29-kDa protein which is absent in subgroup I viruses (Fig. 1).

ORF0

ORF0, present only in subgroup II viruses, encodes a 28-29-kDa protein, the function of which is not clearly known. However, it has been speculated that this protein determines the host range of BWYV (Veidt *et al.*, 1992). van der Wilk *et al.*, (1993) suggested that ORF0 may be associated with symptom expression as transgenic potato

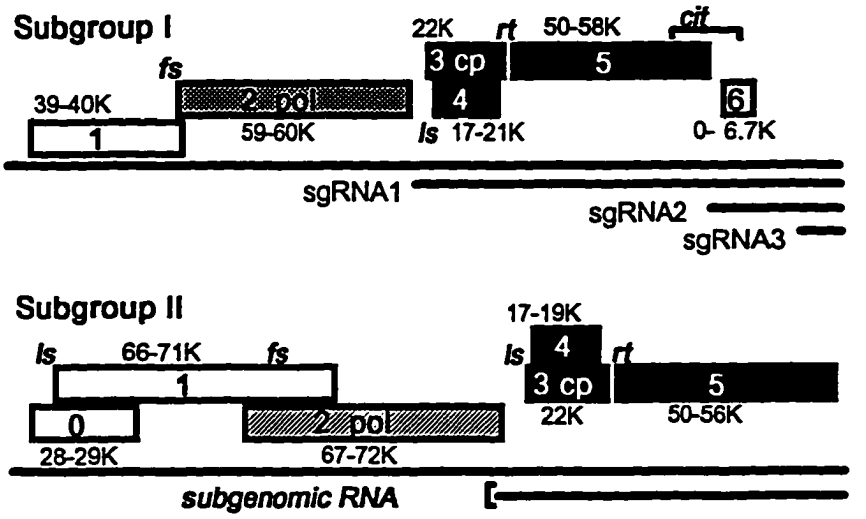


FIG. 1 Genome organizations of luteoviruses (from Miller *et al.*, 1995). Boxes indicate open reading frames (ORFs), with coding capacity shown in kilodaltons (K). Solid black ORFs are conserved between subgroups.Unshaded ORFs have little similarity Subgroup I ORF2 (cross-hatched) is similar to those of carmoviruses. Subgroup II ORF2 (diagonally shaded) is similar to those of sobemoviruses. Abbreviations used: pol = polymerase; fs = frameshifting; cp = coat protein; ls = leaky scanning; rt = readthrough; cit = cap-independent translation.

plants expressing only ORF0 of PLRV showed classic disease symptoms. There is no significant homology in ORF0 between different subgroup II luteoviruses (Demler and de Zoeten, 1991). ORF0 was shown to be dispensable for BWYV replication in protoplasts (Veidt *et al.*, 1992).

ORFs 1 and 2

The putative RNA-dependent RNA polymerase (RdRp; replicase) of all luteoviruses is encoded by ORF2. ORF2 contains a highly conserved amino acid sequence, GXXXTXXXN (X_{25-40}) GDD, a signature sequence of all RdRps (Kamer and Argos, 1984; Habili and Symons, 1989). ORF2 probably does not act alone, because it is expressed via a -1 ribosomal frameshift that occurs just before the termination of translation of ORF1 (Brault and Miller, 1992), resulting in a 99-kDa fusion product (Di *et al.*, 1993). The polymerase ORFs in BWYV (Veidt *et al.*, 1992) and PLRV (Prufer *et al.*, 1992) genomes are also expressed by -1 frameshifting. Helicase motifs, involved in unwinding of double stranded templates, have been identified in ORFs 1 and 2 (Habili and Symons, 1989). However, Koonin and Dolja (1993) dispute this.

The polymerase genes (ORF2) of subgroup I luteoviruses are most closely related to those of the diantho-, tombus-, necro- and carmoviruses. In contrast, the polymerases of subgroup II are similar to those of sobemoviruses (Koonin and Dolja, 1993; Miller *et al.*, 1995). ORFs 1 and 2 have been shown to be essential for the replication of BWYV (Reutenauer *et al.*, 1993) and BYDV-PAV (Mohan *et al.*, 1995) in protoplasts. The

requirement of ORF1 contrasts with the report of Young *et al.* (1991), in which a frameshift mutation near the 5' end of ORF1 did not destroy infectivity.

ORF3

ORF3 of both subgroups encode a 22-kDa polypeptide that functions as coat protein (CP). CP genes of BYDV-PAV (Miller *et al.*, 1988b), BWYV (Veidt *et al.*, 1988) and PLRV (Smith and Harris, 1990) were identified by comparing its amino acid sequence to that of peptides derived from purified virus, by immunoprecipitation of cell-free translation products, and by using anti-CP antibodies to ORF3 expressed in *Escherichia coli.* respectively. CP plays an important role in virus transmission and vector specificity. Even though CP is unnecessary for replication of BWYV (Reutenauer *et al.*, 1993) and BYDV-PAV (Mohan *et al.*, 1995), it is required for formation of intact virus particles and for genomic RNA stability. CP, ORF4 and ORF5 are all expressed from the 3.0kb subgenomic RNA1 (sgRNA1; Dinesh-Kumar *et al.*, 1992; Kelly *et al.*, 1994).

ORF4

Contained within ORF3, but in a different reading frame, is ORF4 which encodes a 17-kDa protein (Fig. 1). ORF4 is expressed by the leaky scanning mechanism of translation initiation (Dinesh-Kumar and Miller, 1993), in which ORF4 is expressed higher than ORF3 (CP) as the start codon of ORF4 is in a better optimal sequence context. Initiation at the ORF4 AUG of PLRV was seven times higher than that at the ORF3 AUG

(Tacke *et al.*, 1993). The protein encoded by ORF4 has biochemical properties of a cell-to-cell movement protein. The carboxy(C)-terminal domain of PLRV 17-kDa protein binds single stranded nucleic acids in a co-operative and sequence non-specific manner (Tacke *et al.*, 1991), similar to that of the movement protein of tobacco mosaic virus (Citovsky *et al.*, 1990). This protein also has the ability to be phosphorylated (Tacke *et al.*, 1993). ORF4 is essential for systemic infection and thus possibly movement in plants (Chay *et al.*, 1996).

ORF5

ORF5 of BYDV-PAV encodes a 50-kDa protein which is expressed as a 72-kDa fusion protein by translational readthrough of the ORF3 amber termination codon (Dinesh-Kumar *et al.*, 1992; Cheng *et al.*, 1994; Filichkin *et al.*, 1994; Wang *et al.*, 1995; Brown *et al.*, 1996). Similar CP readthrough products have been described for other luteoviruses (Veidt *et al.*, 1988; Bahner *et al.*, 1990; Tacke *et al.*, 1990). The readthrough protein is essential for aphid transmission of BWYV (Brault *et al.*, 1995) and BYDV-PAV (Chay *et al.*, 1996). However, this protein is not required for replication or encapsidation of BWYV (Reutenauer *et al.*, 1993) and BYDV-PAV (Filichkin *et al.*, 1994).

ORF6

ORF6, which is present only in subgroup I BYDV's encodes a 4.3-6.7-kDa protein. It can be translated *in vitro* from sgRNA2 (Kelly *et al.*, 1994). SDV (subgroup I

luteovirus) lacks ORF6 and any others 3' of ORF5 (Rathjen *et al.*, 1994). The 5' half of ORF6 is highly conserved between BYDV-PAV and MAV, while the 3' half of ORF6 is the most variable portion of the 3' region among 10 geographically different PAV-like isolates of BYDV (Chaloub *et al.*, 1994). In the full-length infectious PAV6 clone used in our laboratory, ORF6 encodes a 6.7-kDa protein and not a 4.3-kDa protein as reported for 10 different BYDV-PAV isolates (Chaloub *et al.*, 1994). Young *et al.* (1991) reported that a frameshift mutation in ORF6 eliminates viral infectivity, suggesting a role for this gene product in virus replication. However, a BYDV-PAV mutant in which the AUG start codon was altered replicated efficiently in protoplasts, indicating that the ORF6 protein is not necessary for PAV replication (Mohan *et al.*, 1995). A sequence near the ORF6 region that ranges from the 3' end of ORF5 to the 5' end of ORF6 was shown to stimulate translation initiation at the ORF1 AUG from uncapped BYDV-PAV genomic RNA by more than 30-fold *in vitro* (Wang and Miller, 1995).

Genetically engineered virus resistance

Plant viruses cause significant yield losses of important food and fiber crops worldwide. Genetically engineered resistance is desired because natural resistance in many crop plants is inadequate. Several different strategies have been used to stop these harmful viruses. In the recent years, one strategy which has been proven successful in protecting crop plants against viruses is pathogen-derived resistance (PDR). Sanford and Johnston (1985), who proposed the concept of PDR suggested that pathogen genes, when

expressed by a potential host organism, could render that organism resistant to the pathogen. For example, a transgenic plant expressing either a wild-type or modified plant virus gene or gene product may confer resistance by disrupting the pathogen's normal replication cycle. Herskowitz (1987) expanded on this PDR concept in proposing a *trans* dominant negative mutation model. In each model, the expressed exogenous (wild-type or mutant) gene product is predicted to interfere with the normal endogenous or viral gene product.

Genetically engineered resistance has been reported for numerous plant RNA viruses on the basis of the transformation of plants with a pathogen derived gene (for reviews see Wilson, 1993; Scholthof *et al.*, 1993; Fitchen and Beachy, 1993). These studies involved sequences generated from viral coat protein (CP) gene (Powell-Abel *et al.*, 1986), the viral replicase gene (Golemboski *et al.*, 1990) and the viral movement protein gene (Cooper *et al.*, 1995).

Coat protein-mediated resistance

Genetically engineered resistance to a plant virus was first reported by Powell-Abel *et al.* (1986), who observed a delay in symptom development when transgenic tobacco plants expressing the tobacco mosaic tobamovirus (TMV) CP gene were inoculated with TMV. Subsequently, it was shown that the TMV resistance was CP-mediated (Powell *et al.*, 1990). CP-mediated resistance (reviewed by Beachy *et al.*, 1990; Register and Nelson, 1992) has been demonstrated to be effective against viruses in more

than twelve taxonomic groups, including the luteovirus, PLRV (Kawchuk *et al.*, 1990; 1991; Barker *et al.*, 1992). The cellular and molecular mechanisms responsible for virus resistance are not completely understood but the expression of CP may prevent disassembly and/or movement of the virus. In all reports of plants transformed with a luteoviral CP gene, the expression of CP was very low, and the resistance was not complete.

Replicase-mediated resistance

Several recent reports have described virus resistance resulting from transgenic expression of the putative RNA polymerase (replicase) of plant RNA viruses (for reviews see Carr and Zaitlin, 1993; Baulcombe, 1994). Golemboski *et al.*, (1990) reported that transgenic plants expressing the 3'- portion of the replicase gene of TMV were immune to TMV infection. Replicase-mediated resistance has been reported for plants transformed with wild-type or modified replicase genes of cucumber mosaic cucumovirus (CMV; Anderson *et al.*, 1992), potato virus X (PVX; Braun and Hemenway, 1992; Longstaff *et al.*, 1993) and alfalfa mosaic virus (AlMV; Brederode *et al.*, 1995). Replicase-mediated resistance differs from CP-mediated resistance in that it is (i) more effective (immunity), (ii) useful against a narrower range of virus strains and effective against both virion and RNA inoculum. The mechanism(s) of replicase-mediated resistance are not clearly understood. Resistance may be mediated by the polymerase protein, or by the viral RNA itself (Baulcombe, 1994).

Antisense RNA

The use of RNA complementary to part of the viral genome (antisense RNA) is another PDR strategy with great potential. Antisense RNA approaches (reviewed by Bejarno and Lichtenstein, 1992) have been used to generate moderate levels of disease resistance against plant viruses such as tomato golden mosaic geminivirus (Day *et al.*, 1991). PLRV (Kawchuk *et al.*, 1991), brome mosaic bromovirus (BMV; Huntley and Hall, 1993a, b), TMV (Nelson *et al.*, 1993) and cherry leafroll nepovirus (Brooks and Bruening, 1995). However, antisense RNA as an antiviral agent is of low efficiency because of its two major problems:

(1) the large quantity of antisense RNA needed to cope with the replicating system of virus

(2) viral RNA is largely in the cytoplasm, but antisense transcripts are produced in the nucleus (Hull and Davies, 1992)

Thus, it is possible that antisense to an early replicase function to minimize replication, and antisense to a virus with a nuclear phase in its replication cycle are likely to be more effective. The advantage of antisense inhibition is that it can be applied to any gene with great specificity. Antisense RNA might lead to the inhibition of gene expression by forming an RNA-RNA duplex with the sense RNA. The exact mechanism (s) of antisense RNA-mediated resistance are not completely understood.

Sense RNA

Sense RNA approach is an interference strategy used to debilitate genomic viral minus-strand promoter. Sense RNA-mediated resistance has been reported for tobacco etch potyvirus (Lindbo and Dougherty, 1992a, b), PLRV (Kawchuk *et al.*, 1991), BMV (Huntley and Hall, 1993a) and tomato ringspot nepovirus (Yepes *et al.*, 1996). Like antisense RNA, sense RNA can also inhibit gene expression by forming an RNA-RNA hybrid with the minus-strand. Sense RNA can also compete with genomic plus-strand RNAs for minus-strand replicase, resulting in non-productive transcription of complementary minus-strand (Huntley and Hall, 1993a). Swaney *et al.* (1995) suggest that sense RNA-mediated resistance might be a host-mediated response, analogous to selected cases of sense suppression of endogenous genes in transgenic organism.

Virus replication

Deletion and mutation analyses as a tool to study virus replication

In the past few years the genomic RNAs of several plant viruses have been converted into cDNA clones which can be transcribed *in vitro* into infectious RNA molecules. This technique of obtaining the full-length infectious clone has permitted deletion analysis of viral genome. Genes and *cis*-acting elements required for translation, viral replication, virion assembly and movement are identified by deletion and mutation analyses for plant viruses such as BMV (Traynor and Ahlquist, 1990), beet necrotic yellow vein virus (BNYVV; Jupin *et al.*, 1990), AlMV (van der Kuyl *et al.*, 1991a), turnip

crinkle virus (TCV; Hacker *et al.*, 1992) and cymbidium ringspot tombusvirus (CyRSV: Dalmay *et al.*, 1993).

Deletion analysis of a subgroup II luteovirus, BWYV, has identified the viral genes necessary for BWYV replication (Veidt *et al.*, 1992; Reutenauer *et al.*, 1993). Veidt *et al.* (1992) have shown that the expression of BWYV ORF1 and the C-terminal portion of ORF6 are not required for infection of protoplasts. Later, Reutenauer *et al.* (1993) showed that the ORFs 2 and 3 of BWYV, which encode the putative polymerase gene are essential for viral replication. Various deletions in the 3'-part of the BWYV genome within ORFs 4, 5, and 6 did not affect viral replication in protoplasts (Reutenauer *et al.*, 1993). Similar observation has been reported for TCV (Hacker *et al.*, 1992) and CyRSV (Dalmay *et al.*, 1993), in which the two 3'-proximal ORFs are not essential for virus replication.

The effects of some mutations in a subgroup I luteovirus, BYDV-PAV, have been reported by Young *et al.* (1991). However, the recent results obtained in our laboratory (Dinesh-Kumar, 1993; Mohan *et al.*, 1995) and others (Reutenauer *et al.*, 1993; Filichkin *et al.*, 1994) are in contrast to the results of Young *et al.* (1991).The necessity of ORF6 in BYDV-PAV for replication was reported by Young *et al.* (1991). Recently, Mohan *et al.* (1995) have shown that ORF6 is dispensable for BYDV-PAV replication but the *cis*-acting RNA signals in and around ORFs 5 and 6 are necessary for virus replication. The readthrough protein is not essential for replication of BYDV-PAV (Filichkin *et al.*, 1994;

Mohan *et al.*, 1995) and BWYV (Reutenauer *et al.*, 1993). Once again, these results are in contrast with the report of Young *et al.* (1991).

Mutations in the replicase and coat protein genes

Mutations in the putative replicase genes eliminated infectivity of BWYV (Reutenauer *et al.*, 1993) and BYDV-PAV (Mohan *et al.*, 1995), indicating the necessity of replicase gene for luteovirus replication. ORFs encoding the putative replicase genes of many other plant viruses have been shown to be essential for virus replication (Hacker *et al.*, 1992; Dalmay *et al.*, 1993).

Coat protein genes of plant viruses may be involved in viral replication, encapsidation, viral movement and virion assembly. The observation that the protoplasts inoculated with virus having a defective CP gene accumulate less viral RNA than those inoculated with wild-type virus has been reported for BMV (Sacher and Ahlquist, 1989), TMV (Ishikawa *et al.*, 1991), AlMV (van der Kuyl *et al.*, 1991a), PVX (Chapman *et al.*, 1992) and TCV (Hacker *et al.*, 1992). For BMV (Marsh *et al.*, 1991), AlMV (van der Kuyl *et al.*, 1991b) and TMV (Ishikawa *et al.*, 1991), mutations in the CP gene decreased the ratio of positive-strand to negative-strand accumulation in protoplasts, possibly as a result of destabilization of genomic RNA in the absence of encapsidation.

Although CP is dispensable for the replication of BWYV (Reutenauer *et al.*, 1993) and BYDV-PAV (Mohan *et al.*, 1995), it is required for virion assembly and RNA stability. Deletion of a large portion of the CP gene of CyRSV did not prevent replication

of viral RNA, but interfered severly with long-distance translocation (Dalmay *et al.*, 1993). The finding that CP protects and increases genomic RNA accumulation has been shown for BNYVV (Jupin *et al.*, 1990), TCV (Hacker *et al.*, 1992), BWYV (Reutenauer *et al.*, 1993) and BYDV-PAV (Mohan *et al.*, 1995) by encapsidation assays.

Subgenomic mRNAs

The production of subgenomic mRNAs (sgRNAs) is one of the gene expression strategies by which the internally located ORFs of multicistronic eukaryotic RNA viruses may be expressed and regulated during replication. SgRNAs are plus-strand, 3' coterminal copies of genomic RNA, truncated at the 5' end to allow for ribosomal access to each of the internal genes. It has been shown *in vitro* for BMV (Miller *et al.*, 1985) and AlMV (van der Kuyl *et al.*, 1990) and *in vivo* for turnip yellow mosaic virus (Gargouri *et al.*, 1989) that the mechanism of sgRNA synthesis involves internal initiation of transcription, by the viral replicase, on the minus-strand of the virus. Internal initiation is directed by specific subgenomic promoter sequences on the minus-strand. SgRNAs of coronaviruses are believed to be synthesized by discontinuous leader-primed transcription (Lai *et al.*, 1984, 1990).

Three sgRNAs of about 3.0, 0.86 and 0.32 kb have been identified in BYDV-PAV infected tissue or protoplasts (Dinesh-Kumar *et al.*, 1992; Kelly *et al.*, 1994). ORFs 3, 4, and 5 are expressed from the subgenomic RNA1 (sgRNA1). Subgenomic RNA2 (sgRNA2) appears to express ORF6 of BYDV-PAV. The transcription start sites of

sgRNA1 and sgRNA2 of BYDV-PAV occur at a sequence that closely resembles the 5'-end sequence of the genomic RNA (5'-AGUGAAGA) (Kelly *et al.*, 1994). Subgenomic RNA3 (sgRNA3) which is abundantly transcribed and encapsidated by the virus particle, appears to have no coding ability (Kelly *et al.*, 1994). Subgroup II luteoviruses have only one sgRNA with the exception of BWYV in the presence of ST9a RNA.

The 5'-ends of sgRNAs have been mapped for some luteoviruses. Tacke *et al.* (1990) reported the 5'-end of the German isolate of PLRV sgRNA to be at position -40 relative to the CP start codon. Later, Miller and Mayo (1991) mapped the 5'-end of Scottish isolate of PLRV sgRNA to position -212, in the 3'-end of the polymerase gene. Dinesh-Kumar *et al.* (1992) mapped the sgRNA1 transcription start site of an Illinois isolate of BYDV-PAV to base 2769. However, Kelly *et al.* (1994) mapped the sgRNA1 start site to base 2670 in the Australian isolate of BYDV-PAV. Recent results from our laboratory indicates that the sgRNA1 start site is indeed at base 2670 (Mohan and Miller, Chapter 4; G. Koev, unpublished data).

Mapping the subgenomic RNA promoters

Subgenomic RNA promoters of BMV (French and Ahlquist, 1988), CMV (Boccard and Baulcombe, 1993), BNYVV (Balmori *et al.*, 1993), AlMV (van der Vossen *et al.*, 1995), CNV (Johnston and Rochon, 1995), RCNMV (Zavriev *et al.*, 1996) and TCV (Wang and Simon , 1997) have been mapped by deletion and mutation analyses. With the exception of BNYVV sgRNA promoter, most of the plant viral sgRNA

promoters mapped are located primarily upstream of the sgRNA transcription start site. The sgRNA start site and nucleotides immediately flanking it, which are expected to be a part of sgRNA promoter, have been shown to be essential for sgRNA synthesis of AlMV (van der Vossen *et al.*, 1995), CNV (Johnston and Rochon, 1995) and BYDV-PAV (Mohan and Miller, Chapter 4).

Secondary structure in the putative sgRNA promoter region near the sgRNA start site has been identified for red clover necrotic mosaic dianthovirus (RCNMV; Zavriev *et al.*, 1996), TCV (Wang and Simon, 1997) and BYDV-PAV (Mohan and Miller, Chapter 4). Conserved secondary structure might be important for the recognition of the polymerase and the synthesis of sgRNA.

Conserved ACAAA sequence

A conserved ACAAA domain is found at or near the 5'-ends of the genomic RNA and sgRNAs of several members of the luteovirus group and selected related plant viruses (Fig. 2; modified from Miller *et al.*, 1995). Sequence similarity between the 5'-ends of the genomic RNAs and the 5'-ends of sgRNAs has also been reported for many other plant viruses such as BMV (Marsh *et al.*, 1988), AlMV (van der Kuyl *et al.*, 1990), PLRV (Miller and Mayo, 1991), maize chlorotic mottle virus (Lommel *et al.*, 1991) and RCNMV (Zavriev *et* al., 1996). Such sequence similarity may assist the viral replicase in recognizing and interacting with specific minus-strand signals for plus-strand RNA synthesis (Cornelissen *et al.*, 1986).

FIG. 2 Alignments of 5' ends and known and proposed (indicated by ?) subgenomic (SG) RNA transcriptional start sites of luteoviruses and selected related viruses (modified from Miller *et al.*, 1995). Nucleotide positions of the first base of each sequence is indicated by the preceding number. Bold letters indicate similarity between sequences. Known subgenomic start sites are in underlined italics. Dashes indicate gaps added to improve alignments. Abbreviations and references used are as in the text. **A.** Subgroup II luteoviruses and selected other related viruses. **B.** Subgroup I luteoviruses. The start site of BYDV-PAV sgRNA1 at base 2670 (Kelly *et al.*, 1994) is shown. A nine-base tract (CUCACAAAA) located 47 nt downstream of base 2670 is shown because it is identical to bases 17 to 25 near the 5' end of the genome.

A.

SBMV-C-5'	1	C**ACAAAA**UAUAAGA	
SBMV-C-SG?	3240	G**ACAAAA**CCGCGCG	
SBMV-B-SG?	3162	C**ACAAAA**UUAAUUU	Sobemoviruses
RYMV-5'	1	**ACAA**UUGAAGCUA	
RYMV-SG?	3440	C**ACAAA**GAUGGCCA	
PLRVA-5'	1	**ACAAAA**GAAUACC	
PLRVA-SG	3377	**A**CAAAAGAACACU	
BWYV-5'	1	**ACAAAA**GAA-ACC	
BWYV-SG	3259	**A**CAAAAGAU-ACC	Subgroup II
RPV-5'	1	**ACAAA**-GAUUACC	luteoviruses
RPV-SG?	3545	**ACAAAA**CUAAACU	
RPV-SG?	3562	**ACAAAA**CUAGCCG	
RPV-SG?	3575	G**ACAAAC**GUAAGUU	
RCNMV1-5'	1	G**ACAAAC**GUUUUAC	
RCNMV1-SG	2366	A**A**CAAAC**G**UAUUAC	
RCNMV2-5'	1	G**AAAC**CUCGCUC	Dianthoviruses
SCNMV1-SG?	2363	A**ACAAAC**GUAUUAC	
CRSV1-SG?	2297	A**ACAAAC**UUUUUAC	

B.

PAV-5'	1	**AGUGAAGA--UUGAC**CAU-----**CUCACAAAA**
PAV-SG1	2669	U**G**UGAAGG---**UGAC**GA.43nt.**CUCACAAAA**
PAV-SG2	4809	**A**GUGAAGACAACACCACUAGC**ACAAA**U
PAV-SG3	5329	A**UUGAAGA**CG**UU**AAAACUCG-**A**C**GA**CC
PAV-Jpn	1	**AGUGAAGA--UUGAC**CAU-----**CUCACAAAA**
PAV-Jpnsg1	2669	c**G**UGAAGG---**UGAC**GA.43nt.**CUCACAAAA**
SDV-5'	1	**AGUAAAG---UUGAC**ACCUUU**ACAGAA**
SDV-SG?	2731	U**GUAAAGA**GA**UUGAC**GCCUUU**ACUAGA**

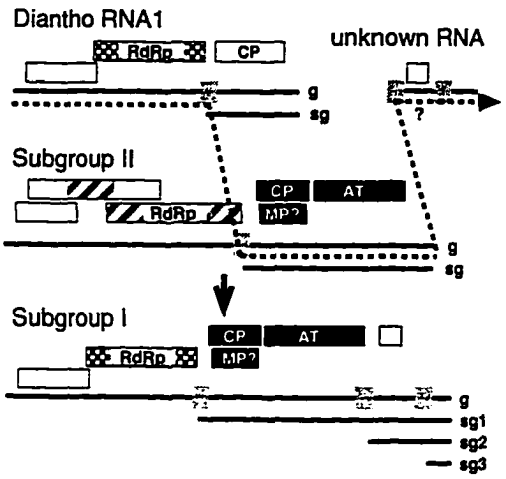
The ACAAA sequence occurs 16 and 19 times in BYDV-PAV and PLRV, respectively. ACAAA motif is very well conserved in subgroup II luteoviruses but not so well conserved in subgroup I luteoviruses (Fig. 2). The minus-strand sequence complementary to the ACAAA motif, perhaps in combination with the U-rich regions, may act as a promoter or enhancer for viral replicase binding and initiation of RNA synthesis (Miller *et al.*, 1995). Mutating the ACAAA domain near the start site of sgRNA of RCNMV abolished sgRNA synthesis (Zavriev *et al.*, 1996). An ACAAA sequence found between bases 2729-2733 of BYDV-PAV, 59 bases downstream of the sgRNA1 transcription start site (2670), might be important for sgRNA1 synthesis.

Luteoviral subgenomic RNA promoters are putative hotspots for viral recombination

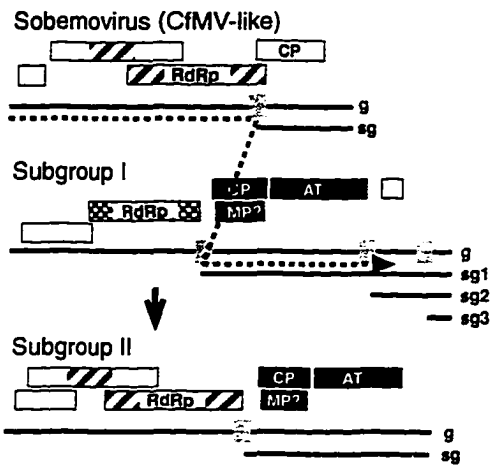
An interesting feature of the divergent origins of the luteoviral genes is that the boundaries where the homology between the two luteovirus subgroups begins and ends occur at sgRNA start sites (Fig. 3). This feature along with the finding that the 5'-ends of genomic RNA and sgRNAs of many luteoviruses are identical, led Miller *et al.* (1995) to propose a recombination model for the origin of luteovirus subgroups, in which the two subgroups arose by polymerase strand-switching at the sgRNA promoters (Fig. 3). For BMV, sgRNA synthesis has been shown to occur by internal initiation of the polymerase on the minus-strand at the subgenomic promoter region (Miller *et al.*, 1985).

FIG. 3 Recombination model for the origin of subgroup I and subgroup II luteoviruses (Miller et al., 1997) Abbreviations used: RdRp = RNA-dependent RNA polymerase; CP = coat protein; MP = movement protein; AT = aphid transmission; g = genomic RNA; sg = subgenomic RNA. Dashed line indicates path of viral polymerase during plus-strand synthesis of the minus-strand template. Arrowhead on dashed line indicates direction of replicase movement and termination site. The nascent strand is never released after strand switches. Strand switches occur only on to the subgenomic RNA promoters (shown as gray boxes). CfMV, cocksfoot mottle virus, is the only sobemovirus with an RdRp translated by frameshifting like all luteoviruses. Model does not explain which subgroup came first, nor where the progenitor originated. Note that at least two recombination events are proposed for subgroup I evolution, but only one is needed (along with some change in ORF size) to generate subgroup II luteoviruses.

Model for Origin of Subgroup I Luteovirus by Recombination



Model for Origin of Subgroup II Luteovirus by Recombination



In this model, in a mixed infection of a diantho-like virus and subgroup II luteovirus, polymerase in the process of synthesizing plus-strand from the diantho-like virus minus-strand could switch strands to the minus-strand subgenomic promoter region of the luteovirus, generating a recombinant progeny RNA with the genome organization of a subgroup I luteovirus (Fig. 3). A similar recombination event with sobemoviral and subgroup I parents could generate a subgroup II luteovirus. Recombination has not been analysed in detail for luteoviruses. Thus, mapping the luteoviral subgenomic RNA promoters will be of great significance because subgenomic promoters are putative origins of replication and putative hotspots for viral recombination (Miller *et al.*, 1995).

Research Goals

The long-term goals of my research were:

1. To obtain genetically engineered resistance to BYDV. Natural resistance is inadequate and thus genetically engineered disease resistance might be very useful for BYDV.
2. To determine the genes and *cis*-acting signals involved in translation, replication and encapsidation of BYDV-PAV RNA as a thorough deletion analysis to determine the necessity and roles of viral genes in replication and encapsidation of subgroup I luteovirus has not been done
3. To map the subgenomic mRNA promoter of sgRNA1 of BYDV-PAV, because subgenomic promoters are putative hotspots of viral recombination and putative origin of replication.

The specific objectives of my research were:-

I. To test the ability of antiviral genes such as antisense RNA, sense RNA, functional replicase and dysfunctional replicase to inhibit BYDV-PAV replication in oat protoplasts.

II. To perform deletion and mutation analyses to identify the roles of each ORF in translation, replication and encapsidation of BYDV-PAV.

III. To identify the correct transcription start site of sgRNA1 by site-directed mutagenesis and to determine the role of conserved ACAAA domain in viral replication.

Dissertation Organization

This dissertation contains three manuscripts. The first manuscript, "Transient expression of antiviral genes of barley yellow dwarf luteovirus-PAV RNA in oat protoplasts", and the third manuscript, "Mutational analysis of the promoter for the barley yellow dwarf luteovirus 3.0-kb subgenomic RNA", are formatted and will be submitted for publication in *Virology*. The second manuscript, "Genes and *cis*-acting sequences involved in the replication of barley yellow dwarf virus-PAV RNA", was published in 1995. *Virology* 212, 186-195. I was principally involved in the research (i.e. experimental design, performing all experiments, data analysis and interpretation) and preparation of all the three manuscripts. In the first manuscript, all the plasmid construction work was done by S. P. Dinesh-Kumar. The contributions of co-authors on the second manuscript were as follows: Most part of Figure 1 was contributed by W. Allen Miller. Figure 2 was

contributed by S. P. Dinesh-Kumar. Figure 3 was jointly done by S. P. Dinesh-Kumar and me. The remaining figures were done by me. W. Allen Miller was also involved in the experimental design, data interpretation and preparation of all the three manuscripts.

In addition to the general introduction chapter and the three manuscripts, this dissertation also includes the general conclusions chapter and two appendices. Appendix 1. "Transient expression of ribozyme as an antiviral agent to barley yellow dwarf luteovirus in oat protoplasts", may be published after some additional work. Appendix 2. "Synergistic interactions between subgroup I and subgroup II luteoviruses", is my contribution to a manuscript, "Are there risks associated with transgenic resistance to luteoviruses?", published in 1997, *Plant Disease* 81, 700-710. The references cited in the general introduction, general conclusions and the appendices are included in the references section at the end of the dissertation.

CHAPTER 2. TRANSIENT EXPRESSION OF ANTIVIRAL GENES OF BARLEY YELLOW DWARF LUTEOVIRUS-PAV RNA IN OAT PROTOPLASTS

A paper to be submitted to Virology

B. R. Mohan, S. P. Dinesh-Kumar and W. Allen Miller

ABSTRACT

Barley yellow dwarf luteovirus (BYDV) is the most economically important virus of barley, wheat and oats, worldwide. Genetically engineered disease resistance to BYDV is essential because natural resistance is inadequate. In this study, we have tested the ability of antiviral constructs such as the sense RNA, antisense RNA, functional replicase and modified replicase genes to inhibit viral replication in oat protoplasts. Sense RNA expressing the 3'-terminal 1450 bases of the BYDV-PAV genome, subgenomic-antisense complementary to the putative subgenomic RNA1 promoter, functional polymerase and modified polymerase (100% frameshift) transcripts, inhibited BYDV-PAV virus accumulation by about 80% in protoplasts as monitored by enzyme-linked immunosorbent assay. However, none of the above constructs decreased viral RNA accumulation in Northern blot analysis. The results presented in this report suggest that targeting BYDV-

PAV genome with viral replicase gene, sense RNA, or antisense RNA might be effective strategies for engineering resistance in plants to BYDV infection.

INTRODUCTION

The concept of pathogen-derived resistance (PDR; Sanford and Johnston, 1985), proposes that the expression of certain genes of pathogen in a potential host organism, would render that host resistant to the pathogen. The report of genetically engineered virus resistance in tobacco plants expressing the coat protein (CP) gene of tobacco mosaic virus (TMV: Powell-Abel *et al.*, 1986), was the first application of PDR concept in plant virology. This type of "coat protein-mediated resistance" has been shown to be effective against several viruses (for reviews see Beachy *et al.*, 1990; Register and Nelson, 1992). PDR, which includes strategies such as use of viral CP gene or the viral replicase gene or viral movement protein gene or the satellite RNA and the antisense RNA sequences, has been proven successful in generating virus-resistant plants (for reviews see Fitchen and Beachy, 1993; Scholthof *et al.*, 1993; Wilson, 1993).

Transformation of plants with genes encoding all or part of the viral replicase (RNA-dependent RNA polymerase) gene has been a successful strategy in the generation of virus-resistant plants. This type of resistance, generally called "replicase-mediated resistance", is highly strain specific and effective against higher levels of virus inoculum, than CP-mediated resistance (for reviews see Carr and Zaitlin, 1993; Baulcombe, 1994). Replicase-mediated resistance has been reported for plants transformed with wild-type

(wt) or modified replicase genes of TMV (Golemboski *et al.*, 1990), pea early browning tobravirus (MacFarlane and Davies, 1992), cucumber mosaic cucumovirus (CMV; Anderson *et al.*, 1992), potato virus X (PVX; Braun and Hemenway, 1992; Longstaff *et al.*, 1993), cymbidium ringspot tombusvirus (CyRSV; Rubino *et al.*, 1993) and alfalfa mosaic virus (AlMV; Brederode *et al.*, 1995).

Antisense RNA approaches have been used to inhibit expression of some plant (van der Krol *et al.*, 1988) and viral (Hirashima *et al.*, 1986) genes. Early reports of transgenic plants expressing antisense RNAs against CP genes of TMV (Powell *et al.*, 1989), PVX (Hemenway *et al.*, 1988) and CMV (Cuozzo *et al.*, 1988), describe only weak or no protection against low concentrations of virus inoculum. However, recently antisense strategies have proved to be more successful as directed against potato leafroll luteovirus (PLRV; Kawchuk *et al.*, 1991), tomato golden mosaic geminivirus (Day *et al.*, 1991), tobacco etch potyvirus (Lindbo and Dougherty, 1992a), brome mosaic bromovirus (BMV; Huntley and Hall, 1993a,b), TMV (Nelson *et al.*, 1993) and cherry leafroll nepovirus (CLRV; Brooks and Bruening, 1995).

Barley yellow dwarf virus (BYDV) is the type member of the luteovirus group of plant viruses (for reviews see Mayo and Ziegler-Graff, 1995; Miller *et al.*, 1995). The PAV serotype of BYDV has a positive-sense RNA genome of ~5.7kb encoding at least six open reading frames (ORFs; Fig. 1). ORFs 1 and 2, located in the 5' half of the genome, have been shown to be essential for replication in oat protoplasts (Mohan *et al.*, 1995). ORF2, encoding the 60-kDa protein, a putative RNA-dependent RNA polymerase

(replicase), is expressed by a translational frameshift event (Brault and Miller, 1992), resulting in a 99-kDa fusion protein. The 3' half of the genome is expressed by three subgenomic RNAs (Kelly *et al.*, 1994). ORF 3 encodes the 22-kDa viral CP. ORF5 encodes a 50-kDa protein which is expressed as a 72-kDa fusion protein by translational readthrough of the ORF 3 amber termination codon (Dinesh-Kumar *et al.*, 1992; Brown *et al.*, 1996). ORF 6 which has no known function encodes a 4.3-6.7-kDa protein. BYDV is the most widespread and economically important virus of small grain cereals such as oats, barley and wheat, worldwide (D'Arcy and Burnett, 1995). Because of the lack of natural disease resistance genes in many small grain crops, genetically engineered resistance would be greatly useful for BYDV.

In this paper, we have tested the ability of potential antiviral constructs such as sense RNA, antisense RNA, wild-type replicase and modified replicase to inhibit BYDV-PAV replication in oat protoplasts, as monitored by enzyme-linked immunosorbent assay (ELISA). This work leads toward our goal of obtaining genetically engineered disease resistance to BYDV in plants.

MATERIALS AND METHODS

Plasmid Construction

The construction of plasmids pPAV6 and pFLFSM4 have been described by Di *et al.* (1993). Construction of plasmids pSP9, pSP10 and pSP15-1 have been described previously (Dinesh-Kumar *et al.*, 1992; Dinesh-Kumar, 1993). Mohan *et al.* (1995) have

described the construction of plasmid pPAV1-1. With the exception of PAV6 and pGEM7Z, all antisense (AS) or sense (S) transcripts are named for the region of the PAV6 genome to which they are targeted. Base numbering refers to the BYDV-PAV genome as in Miller *et al.* (1988).

Transcripts produced from plasmids pPAV1-1, pSP15-1, pSP9 and pSP6 were termed 5'-antisense (5'-AS), frameshift-antisense (FS-AS), subgenomic-antisense (SG-AS) and coat protein-antisense (CP-AS), respectively. Plus- and minus-strand transcripts obtained from plasmid pSP10 were called 3'-sense (3'-S) and 3'-antisense (3'-AS), respectively. Transcript produced from plasmid pFLFSM4 was named 100% frameshift (100%FS) and POL indicates transcript of wild-type, fuctional polymerase.

***In vitro* transcription**

All plasmids were linearized with a particular restriction enzyme prior to transcription. RNAs were transcribed with T7 or SP6 RNA polymerase using Megascript kit (Ambion, Austin, TX). Final RNA concentration was determined spectrophotometrically. ^{32}P-labeled pSP9 antisense probe was synthesized by *in vitro* transcription as described by Promega (Madison. WI) using [α^{32}P]-CTP. pSP9 was linearized with EcoICRI prior to transcription with SP6 RNA polymerase.

Plasmids pPAV1-1, pSP6 and pSP9 were linearized with EcoRI, BamHI and EcoRICRI, respectively, prior to transcription with SP6 RNA polymerase. Plasmids pPAV6 and pSP15-1 were linearized with SmaI and HindIII, respectively, prior to

transcription with T7 RNA polymerase. Transcription of SmaI linearized pSP10 with SP6 RNA polymerase gave plus-strand SP10, whereas transcription of HindIII linearized pSP10 with T7 RNA polymerase gave minus-strand SP10 transcript.

Protoplast inoculation and RNA extraction

Protoplasts were isolated from *Avena sativa* cv Stout suspension culture (cell line S226 obtained from Howard Rines, USDA/ARS, University of Minnesota) as described previously (Dinesh-Kumar and Miller, 1993). Total RNA was isolated from inoculated protoplasts with a small-scale procedure that uses aurintricarboxylic acid as an RNase inhibitor (Wadsworth *et al.*, 1988; Dinesh-Kumar and Miller, 1993).

Northern blot analysis

RNA was electrophoresed on a 1% denaturing agarose gel containing formaldehyde, blotted to nylon membrane (Gene Screen, DuPont) and hybridized with ^{32}P-labeled antisense RNA probe as described in Mohan *et al.* (1995). Blots were dried and exposed to X-ray film with an intensifying screen at -80°.

ELISA

ELISA plates were coated with BYDV-PAV-specific polyclonal antibodies for three hours at 37° and blocked with 1% fat-free carnation dry milk solution. Plates were incubated with antigen (protoplast extract) overnight at 4°. The following day plates were

washed three times (three minutes each), with phosphate-buffered saline containing 0.1% tween. Alkaline phosphatase-PAV polyclonal antibody conjugate was loaded onto the plates and incubated for three hours at 37°. Plates were washed as above, and the substrate was added to the plates. ELISA readings (A_{405}) were taken using Dynatech minireader.

RESULTS

Effect of sense and antisense RNA on BYDV-PAV replication

Antisense (AS) and sense (S) RNA constructs targeted to different regions of BYDV-PAV genome are shown in Fig. 1B. PAV6, the full-length infectious, wt transcript was co-electroporated with 25-fold molar excess of the S and AS transcripts. After 48 hours, protoplasts were harvested and virion accumulation was monitored by ELISA using antibody raised against virions. Positive controls such as viral RNA and PAV6 (NO AS) gave high ELISA readings (A_{405} = 1.2).

5'-AS (complementary to bases 1-546 of BYDV-PAV) and 3'-AS (complementary to 1450 bases from 3' end of BYDV-PAV) gave 37% and 52% inhibition of virus accumulation, respectively, in oat protoplasts (Fig. 2B). Antisense RNA to 5' or 3' region of other plant viruses has been shown to inhibit viral replication (Nelson *et al.*, 1993; Huntley and Hall, 1993a; Brooks and Bruening, 1995). FS-AS did not inhibit virus accumulation, as it gave high ELISA values similar to that of viral RNA alone (A_{405} = 1.2 for FS-AS and viral RNA). 54% inhibition was obtained by CP-AS (antisense to bases 2985 - 3465). These results concur with previous observations of Kawchuk *et al.* (1991),

Brooks and Bruening (1995) and Yepes *et al.* (1996) for PLRV, CLRV and tomato ringspot nepovirus, respectively, in which CP-AS reduced viral accumulation. Interestingly, SG-AS (antisense to bases 2737 - 2985 of BYDV-PAV) gave 75% inhibition (A_{405} = 0.2) of BYDV-PAV virus accumulation (Fig. 2B). Similar inhibition percentages were observed for all of the above constructs in two independent protoplast experiments.

3'-S (sense-strand expressing 1450 bases from 3' end of BYDV-PAV) reduced BYDV-PAV ELISA readings by 63% (A_{405} = 0.18; Fig. 2B). A similar observation was noted for 3'-S RNA of BMV (Huntley and Hall, 1993a). Evidence that the decrease in ELISA values in presence of added (+)-sense RNA does not result from reduced uptake of inoculated viral RNAs by the oat protoplasts at higher inoculum levels is provided by the results shown in Fig. 2B, where a 25-fold molar excess of pGEM7Z RNA, coelectroporated with PAV6 gave ELISA values (A_{405} = 1.05) similar to that of PAV6 alone (A_{405} = 1.07). These results are similar to those of Rao and Hall (1991) and Huntley and Hall (1993a), who showed that the coinoculation of protoplasts with BMV genomic RNA and excess non-specific RNA had no appreciable effect on viral replication.

Optimum molar excess of antisense constructs

What is the optimum molar excess of sense and antisense constructs needed for efficient inhibition of viral replication in protoplasts? To answer this question, 1, 5, 20 and 50-fold molar excess of SG-AS, 5'-AS, 3'-AS, 3'-S and the control pGEM7Z transcripts

were coelectroporated with PAV6 RNA. After harvesting at 48 hours, virion accumulation was assayed by ELISA (Fig. 3).

With the exception of SG-AS, significant inhibition of virion accumulation was not observed for any constructs either at 1-fold or 5-fold molar excess (Fig. 3). 20-fold molar excess of AS proved to be optimum for inhibition of virus accumulation, as the use of 50-fold molar excess decreased the ELISA value of PAV6 + pGEM7Z (control) drastically. For example, PAV6 + 3'-S gives 7%, 9%, 64% and 58% inhibition at 1, 5, 20 and 50-fold molar excess, respectively. In a similar type of experiment, 22-fold molar excess of AS transcript gave the highest inhibition of BMV RNA accumulation (Huntley and Hall, 1993a).

Northern blot analysis shows no inhibition of BYDV-PAV replication by sense and antisense constructs

AS and S RNA inhibited BYDV-PAV virion accumulation as indicated by low ELISA values. To confirm that this was due to inhibited replication, PAV6 was coelectroporated with 20-fold molar excess of 3'-AS, 3'-S, 5'-AS and SG-AS. After 48 hours, viral RNA replication products were detected by Northern blot hybridization. PAV6 RNA replicated efficiently, giving large amounts of genomic RNA (gRNA) and subgenomic RNA1 (sgRNA1) (Fig. 4; lane 1). 20-fold molar excess of non-specific RNA (pGEM7Z) coinoculated with PAV6 had no significant effect on PAV6 RNA accumulation (Fig. 4; lane 6).

Surprisingly, even in presence of 20-fold molar excess of 3'-AS, 3'-S, 5'-AS or SG-AS RNAs in the inoculum, large amounts of gRNA and sgRNA1 accumulated at levels similar to that when cells were inoculated with PAV6 alone (Fig. 4, compare lanes 2, 3, 4 and 5 with lane 1). There was no decrease in PAV6 RNA accumulation in the presence of added plus or minus-sense RNAs. Reduced levels of PAV6 RNA in lane 5 (Fig. 4) was due to lower loading of RNA on the agarose gel (data not shown). These results suggest that the interference of virus life cycle by sense or antisense RNA might be at the protein level rather than the RNA level.

Effect of functional polymerase and 100%FS on virus accumulation

Functional polymerase (POL) and 100%FS were tested for their ability to inhibit PAV6 virion accumulation in oat protoplasts. In 100%FS , the UAG stop codon of 39K was altered to UCAG (Di *et al.*, 1993), resulting in a 99-kDa fusion protein (Fig. 5A). This construct does not produce 39-kDa protein when translated. POL and 100%FS RNA were coelectroporated with PAV6 RNA. After 48 hours, virus accumulation was monitored by ELISA using antibody against BYDV-PAV virions.

100%FS and POL RNA inhibited PAV6 virus accumulation by 77% (A_{405} = 0.25) and 85% (A_{405} = 0.16), respectively (Fig. 5B). Positive controls (viral RNA and PAV6) gave high ELISA readings (A_{405} = 1.2). Coinoculation of PAV6 with pGEM7Z (non-specific RNA) gave ELISA values (A_{405} = 1.07) similar to that of wt PAV6 (A_{405} = 1.15) (Fig. 5B). In agreement with our results, transgenic tobacco plants expressing wild-type or

modified replicase genes have conferred resistance to several plant viruses (Golemboski *et al.*, 1990; Anderson *et al.*, 1992; Braun and Hemenway, 1992; Audy *et al.*, 1994). However, Northern blot analysis showed no decrease in viral RNA accumulation in the presence of added functional polymerase or 100%FS transcripts (data not shown).

DISCUSSION

Most of the antiviral constructs tested in this study significantly inhibited virus accumulation (as indicated by low ELISA values) in protoplasts but showed no decrease in RNA levels in Northern blot hybridization. This discrepancy between the results of ELISA and Northern blot analysis is difficult to explain because the samples with low ELISA values are generally expected to have reduced RNA levels. Two possible explanations for the inconsistency between the results of ELISA and Northern blot hybridization are: (i) The procedure used for Northern bot hybridization might not be sufficiently quantitative to differentiate the RNA levels from one sample to another. (ii). The antiviral constructs might be interfering with the virus cycle at some step between RNA and virus accumulation. Currently, the exact mechanism by which these antiviral constructs give low ELISA values but no reduced RNA levels is not understood. Mechanisms for these antiviral strategies proposed in other plant viruses are discussed.

Antisense RNA-mediated resistance

Antisense RNAs targeted to different regions of the BYDV-PAV genome inhibited viral accumulation in oat protoplasts as monitored by ELISA (Fig. 2). SG-AS targeted to the putative sgRNA1 promoter inhibited BYDV-PAV virus accumulation by 75%, whereas FS-AS targeted to the frameshift region had no effect on virion accumulation. This indicates that the judicious selection of target sites is essential for the antisense strategy to succeed against viruses as RNA viruses have evolved mechanisms to prevent complementary RNA from blocking replication. FS-AS might not have bound properly to its target site as there is substantial predicted secondary structure near the frameshift region (Brault and Miller, 1992). SG-AS, antisense to the intercistronic region 5' of the CP gene, may inhibit by acting as viral replication origin. Because the intercistronic AS spans the region comprising the 5' end of sgRNA1 from which the CP and other 3' ORFs are translated (Kelly *et al.*, 1994), it would be expected to have a subgenomic promotor and compete with the infecting viral RNA for replicase. An analogous RNA targeted to the subgenomic promoter of BMV inhibited viral replication (Huntley and Hall, 1993b).

5' and 3' termini and internal promoter binding sites are attractive targets for AS strategy. Inhibition of the replication or translation of the earliest viral gene with AS RNA complementary to one or both of the termini of the viral genomic RNA would likely effect the most complete resistance (Miller and Young, 1995). 5'-AS and 3'-AS reduced virus titre by 37% and 52%, respectively (Fig. 2B). Similar 5' or 3'-AS constructs inhibited replication of TMV (Nelson *et al.*, 1993) and CLRV (Brooks and Bruening, 1995). CP-

AS, complementary to the multifunctional CP gene, gave 54% inhibition of PAV6 replication. Analogous CP-AS RNA has been shown to inhibit replication of other plant viruses (Kawchuk *et al.*, 1991; Brooks and Bruening, 1995; Yepes *et al.*, 1996).

The exact mechanism(s) of AS RNA-mediated resistance are not clearly known. The formation of an RNA-RNA duplex between sense and antisense RNA might lead to the inhibition of gene expression. AS RNA-based resistance may be due to the prevention of ribosome binding for translation, the inhibition of the transport from the nucleus in the case of nucleus-encoded genes, or the creation of a substrate for double strand-specific nucleases. Optimizing the binding of AS RNA, extending the half-life of AS RNA and expressing high levels of AS RNA are the key factors for the success of antisense strategy.

Sense RNA as an antiviral strategy

Morch *et al.* (1987) from their *in vitro* replicase experiments with turnip yellow mosaic virus showed that the 3' sense RNA fragments compete with genomic (+) strand RNAs for (-) strand replicase, resulting in non-productive transcription of complementary (-) strand fragments. Our result shown in Fig. 2B, where in the 3'-S inhibited 83% of virus accumulation, confirms the value of sense RNA as an antiviral strategy. This type of strategy also interfered with BMV RNA replication (Huntley and Hall, 1993a).

Sense RNA has the ability to form RNA-RNA hybrids with (-) strand RNAs and to compete effectively with viral genomic RNAs both for host factors and, especially, for the limited pool of viral replicase (Huntley and Hall, 1993a). Since the viral (-) strand RNA is

present in much lower concentrations than (+) strand RNA, low concentrations of the complementary RNA would be required to cause interference. Therefore, the sense RNA could make a better antiviral strategy than the AS RNA. It has been proposed that the untranslatable or truncated sense RNA (Lindbo and Dougherty, 1992a,b) could provide better protection because, unlike the translatable transcript, it is not associated with ribosomes and thus better accessible for interacting with the (-) strand RNA.

Replicase -mediated resistance

Transgenic tobacco plants expressing the wild-type replicase genes have conferred resistance to PVX (Braun and Hemenway, 1992), CyRSV (Rubino *et al.*, 1993) and PVY (Audy *et al.*, 1994). Our result with POL (expressing the wt replicase gene) is consistent with the above observations, as POL inhibited virion accumulation by 85% in protoplasts (Fig. 5B). However, transgenic plants expressing AlMV (Taschner *et al.*, 1991) or BMV (Mori *et al.*, 1992) functional replicase genes did not exhibit resistance, but instead, complemented replicase-defective mutants. 100%FS inhibited virus accumulation by 77% in protoplasts. Transgenic plants expressing modified replicase genes have conferred resistance in many plant viruses (Anderson *et al.*,1992, Longstaff *et al.*,1993, Audy *et al.*, 1994 Brederode *et al.*,1995). In most of the above examples, the modified replicase genes are truncated or mutated to make it dysfunctional. However, in 100%FS, the replicase is modified but not quite dysfunctional.

The mechanism(s) underlying replicase-mediated resistance are not yet clear. It was suggested (Anderson *et al.*, 1992) that the resistance may result from a form of "*trans*-dominant negative mutation interference mechanism" (Herskowitz, 1987). In this mechanism, the expressed exogenous (wt or mutant) gene product is predicted to interfere with the normal endogenous or viral gene product. Longstaff et al (1993) have suggested many routes through which the modified replicase can interfere with virus replication. Transgenic plants expressing the defective replicase gene of CMV has been shown to suppress both the viral replication (primary effect) and the long-distance virus movement (secondary effect) (Carr *et al.*, 1994; Hellwald and Palukaitis, 1995). At this stage, it is not clear how the replicase-mediated resistance works at the molecular level against BYDV-PAV replication.

Protoplasts vs transgenic plants

In this report, we have tested the ability of antiviral strategies to inhibit BYDV-PAV virion accumulation in oat protoplasts. The protoplast system has the attraction of allowing constructs that are ineffective in reducing virus titre in a transient assay to be eliminated from further consideration or to be given lower priority for testing in transgenic plants. Our results from antiviral constructs, may underestimate the protection achievable in transgenic plants, because some protoplasts that did not receive antiviral construct may have received wt PAV6 RNA. Also, antiviral and viral RNA arrive at the same time in protoplasts, but constitutively expressed antiviral gene would already be present at higher

levels in transgenic plants than the invading viral RNA. The natural infection probably is initiated by only few virus particles, whereas in the protoplast assay, millions of viral genome are present per cell. Additionally, the expression of the antiviral sequences is transient in protoplasts rather than constitutive, as it would be in a transgenic plant. The idea that the results from protoplasts may underestimate the capabilities of the same antiviral construct in transgenic plants is supported by the results of Golemboski *et al.* (1996) and Carr *et al.* (1992).

SG-AS, POL and 100%FS which gave significant inhibition (~80%) of virus accumulation in oat protoplasts have been introduced into transgenic oat plants in collaboration with Dr. Somers (University of Minnesota). Screening of these transgenic plants for BYDV resistance will shed more light on antisense RNA-based resistance and replicase-mediated resistance.

ACKNOWLEDGMENTS

The authors thank Dr. S. P. Dinesh-Kumar for construction of the plasmids and the laboratory of Dr. Somers (University of Minnesota) for generation of transgenic plants. This work was funded by grants from the Quaker Oats Company, the Consortium for Plant Biotechnology Research and USDA Biotechnology Risk Assessment Grant 94392100531. This is paper No. J- of the Iowa State University Agricultural and Home Economics Experiment Station.

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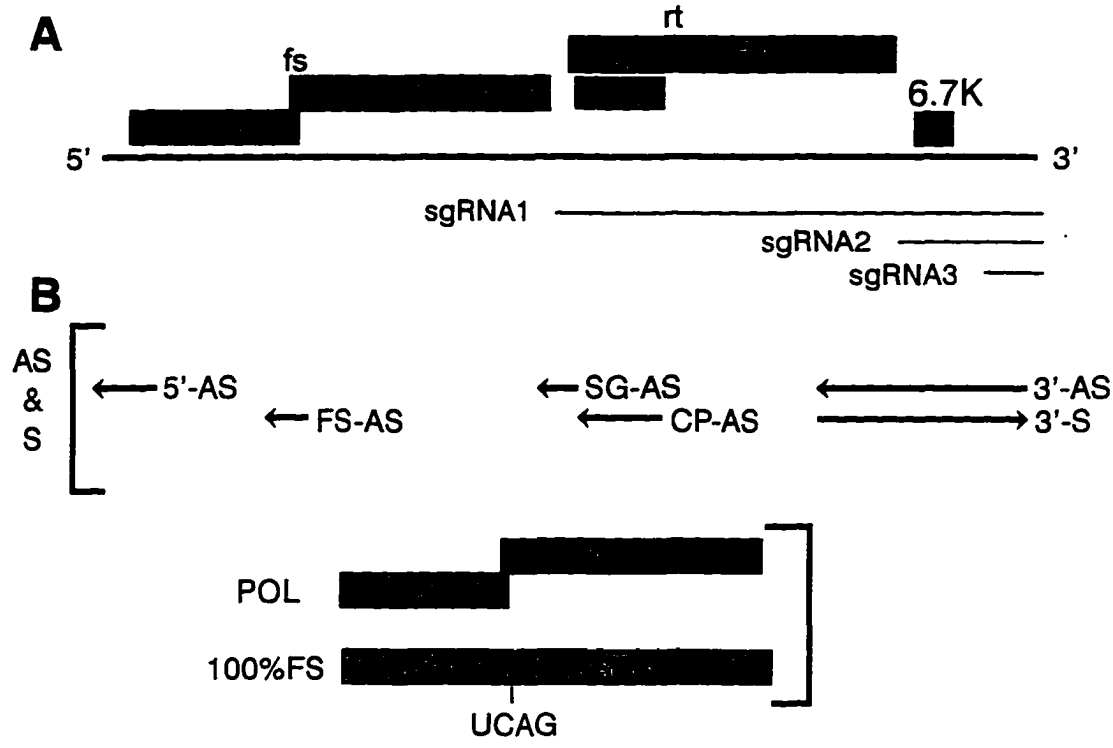
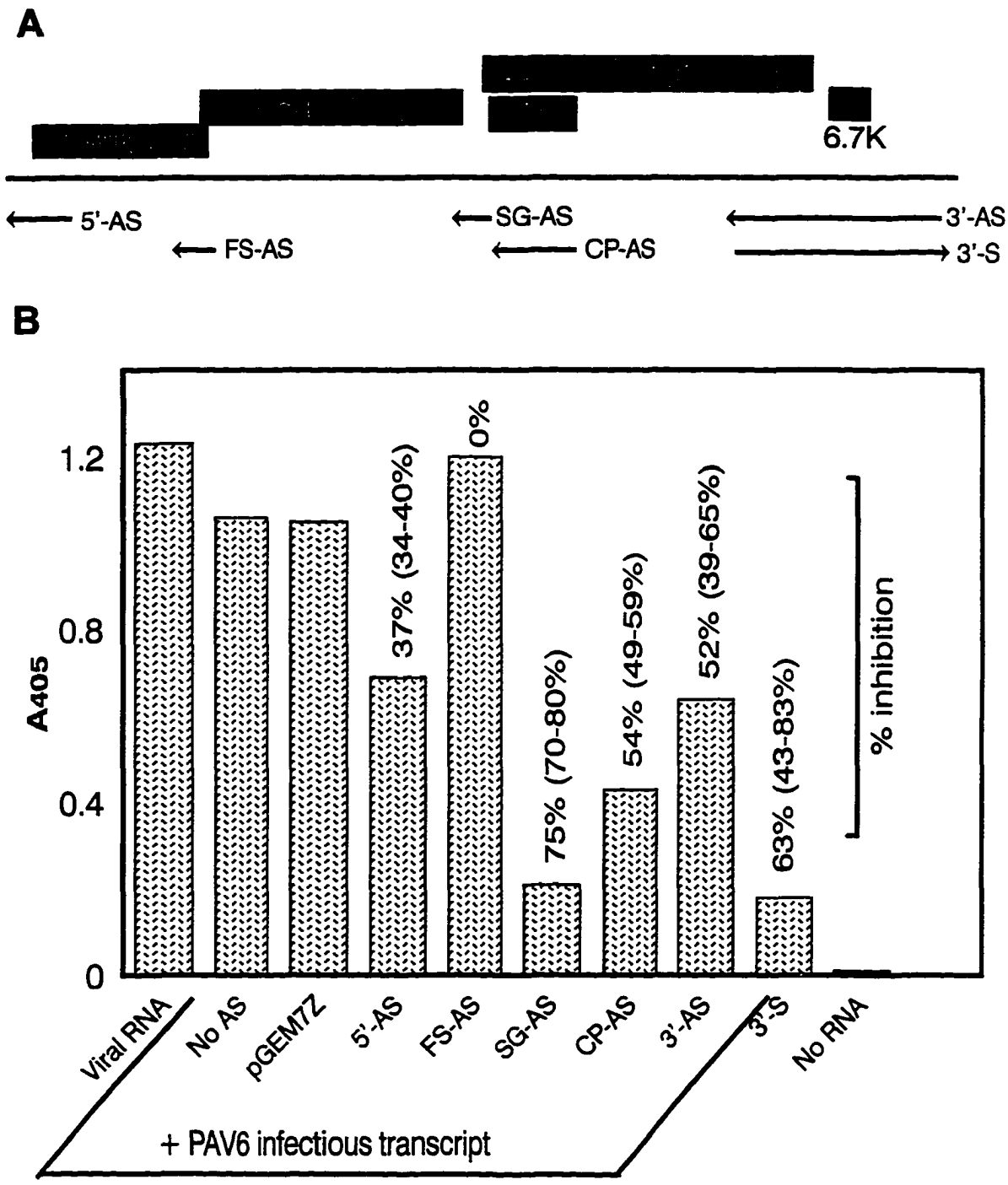


FIG. 1 (A) Genome organization of BYDV-PAV. Open reading frames (ORFs) are indicated by the size of protein product inside the box. Lines below ORFs indicate genomic and subgenomic RNAs. Abbreviations used: fs = frameshift; rt = readthrough. (B) Transcripts tested as antiviral constructs to BYDV-PAV genome. Abbreviations used are as follows: AS = antisense; S = sense; FS-AS = frameshift-antisense; SG-AS = subgenomic-antisense; CP-AS = coat protein-antisense; POL = functional polymerase; 100%FS = 100% frameshift. Arrows indicate the position and orientation of S and AS RNAs.

FIG. 2 (A) Sense and antisense RNAs targeted to different regions of BYDV-PAV genome. Abbreviations used are as follows: AS = antisense; S = sense; FS-AS = frameshift-antisense; SG-AS = subgenomic-antisense; CP-AS = coat protein-antisense. Arrows indicate the position and orientation of S and AS RNAs. (B) Antisense and sense RNA inhibition of BYDV-PAV virus accumulation in oat protoplasts. 10μg of wild-type PAV6 RNA was coelectroporated with 25-fold molar excess of S or AS RNAs indicated below each bar. After 48 hours, protoplasts was harvested and virus accumulation was monitored by ELISA using antibody raised against PAV virions. ELISA readings (A_{405}) are shown on the Y-axis. Average inhibition % resulting from two independent protoplast experiments (each done in duplicate) is shown with the range in parentheses. Abbreviations used are as in Fig. 2A.


A
6.7K
5'-AS
FS-AS
SG-AS
CP-AS
3'-AS
3'-S
B
A405
1.2
0.8
0.4
0
37% (34-40%)
0%
75% (70-80%)
54% (49-59%)
52% (39-65%)
63% (43-83%)
% inhibition
Viral RNA
No AS
pGEM7Z
5'-AS
FS-AS
SG-AS
CP-AS
3'-AS
3'-S
No RNA
+ PAV6 infectious transcript

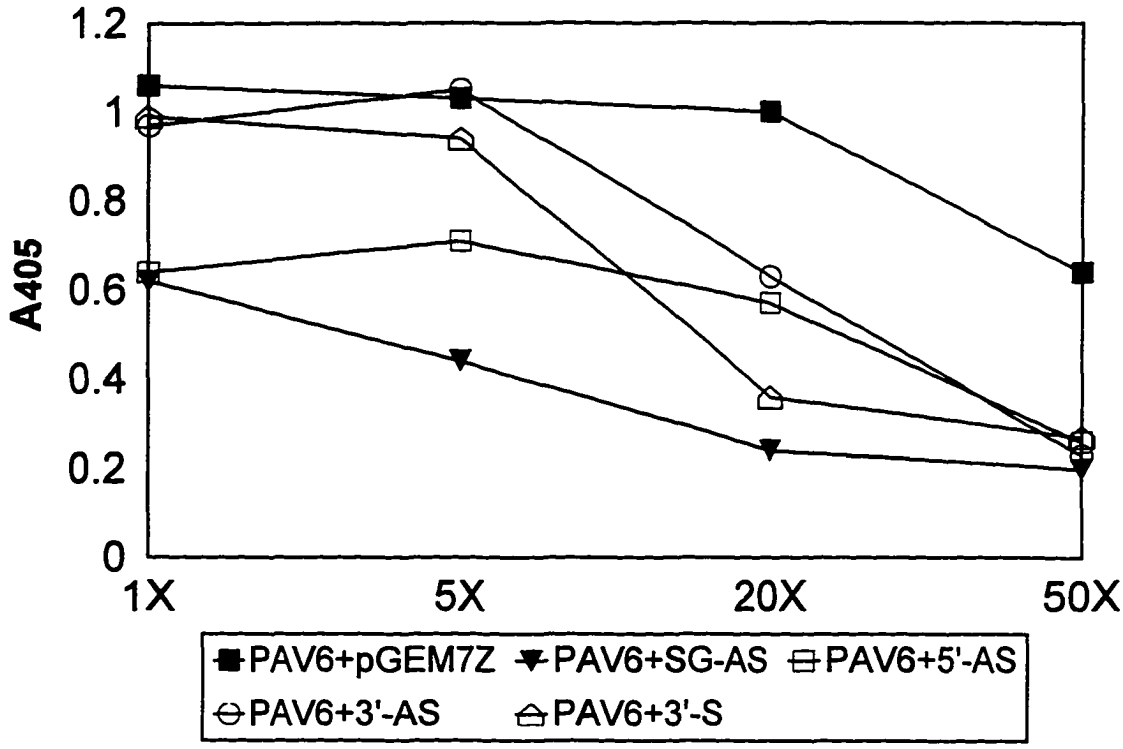


FIG. 3 Optimum molar excess of sense (S) and antisense (AS) RNAs. 10μg of wild-type PAV6 RNA was coinoculated with 1, 5, 20 and 50-fold molar excess (shown on the X-axis) of S or AS RNAs. After 48 hours, protoplasts was harvested and viral accumulation was assayed by ELISA. ELISA values (A_{405}) are shown on the Y-axis. Abbreviations used are as in Fig. 2A.

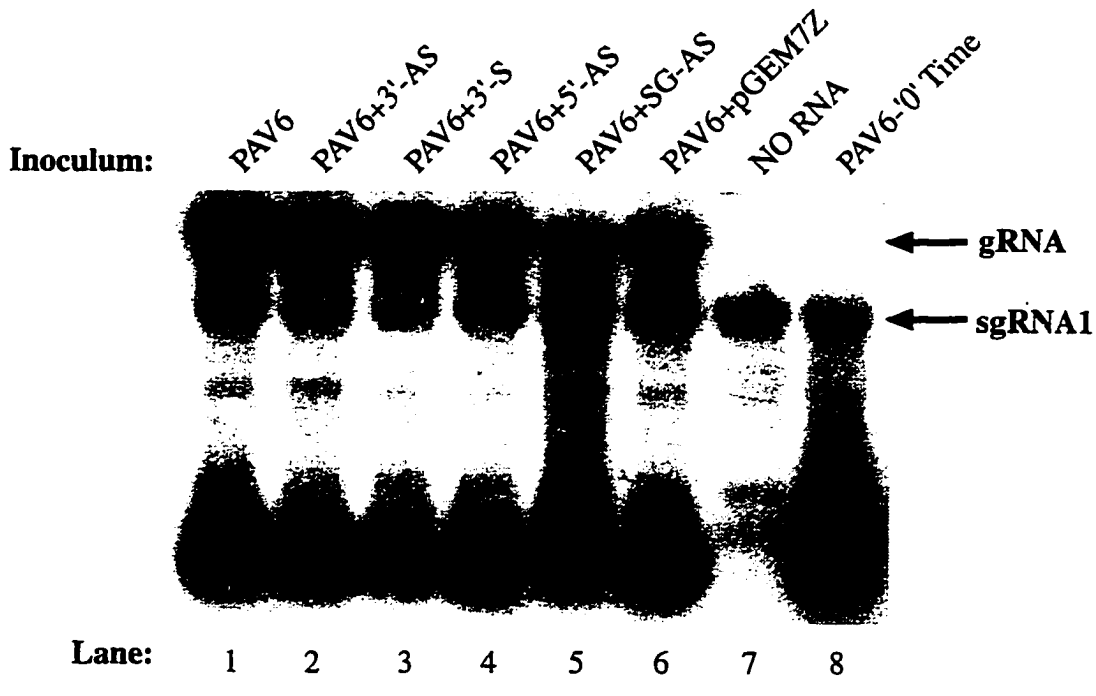
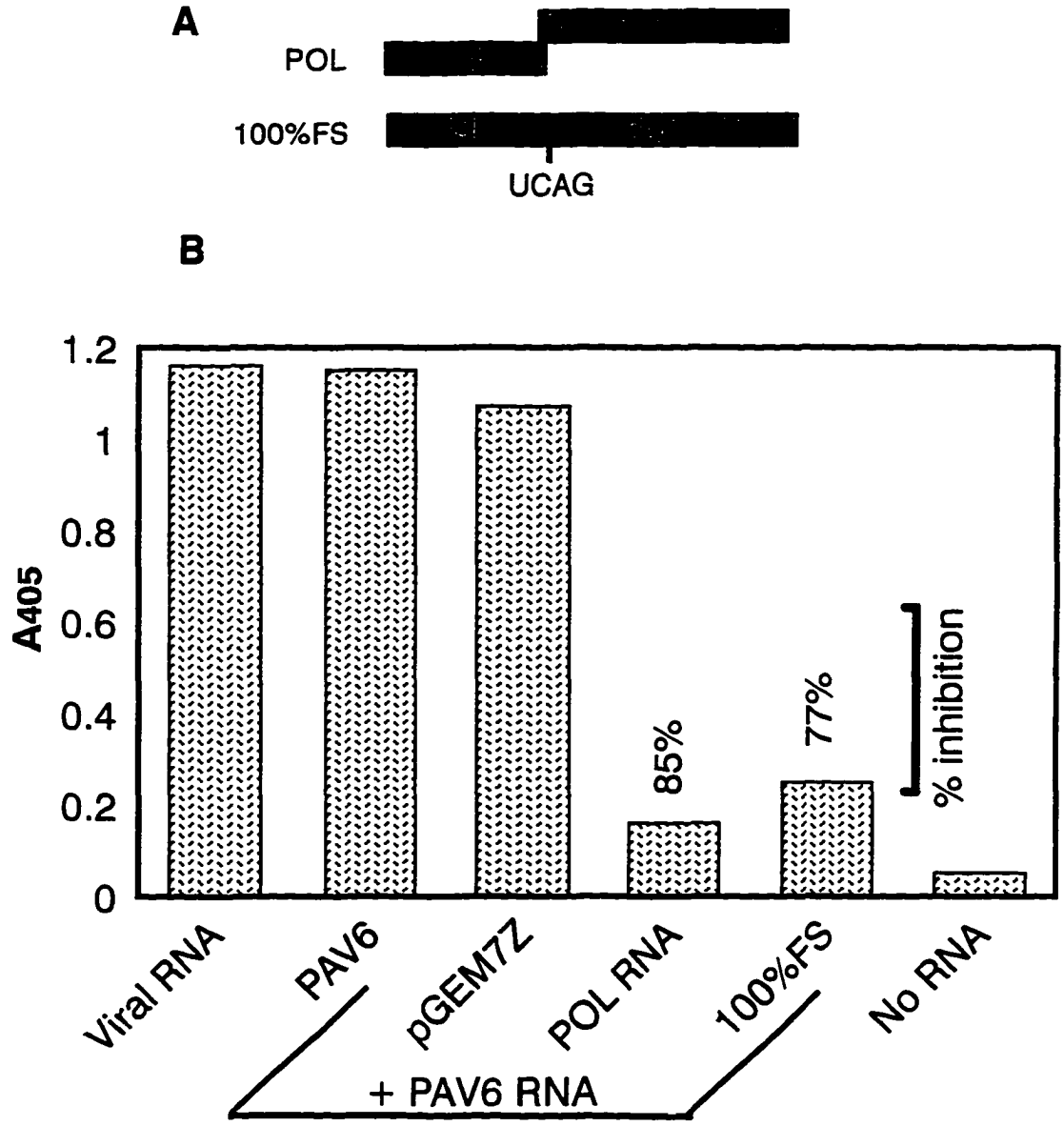


FIG. 4 Northern blot hybridization of RNA from cells inoculated with mixtures of antisense (20-fold molar excess) and wild-type PAV6 transcripts. Total RNA was extracted from protoplasts 48 hours after inoculation with the transcripts indicated above each lane. Blots were probed with transcript from pSP9, complementary to the bases 2737-2985 of BYDV-PAV genome. No RNA indicates total RNA from uninoculated protoplasts. Mobilities of genomic (g) and subgenomic (sg) RNA1 are shown on the right.

FIG. 5 (A) Functional polymerase (POL) and 100% frameshift (100%FS) transcripts of BYDV-PAV. In 100%FS, the UAG stop codon of 39K was altered to UCAG (Di *et al.*, 1993), resulting in a 99-kDa fusion product. (B) Inhibition of viral accumulation in oat protoplasts by functional polymerase and 100%FS. 10μg of wild-type PAV6 RNA was electrporated with 10μg (two-fold molar excess) of POL or 100%FS RNA. 48 hours after inoculation, virion accumulation was monitored by ELISA using antibody raised against PAV virions. ELISA readings (A_{405}) are shown on the Y-axis. Average inhibition % resulting from two replications is shown on top of the vertical bar.


A
POL
100%FS
UCAG
B
A405
1.2
1
0.8
0.6
0.4
0.2
0
85%
77%
% inhibition
Viral RNA
PAV6
pGEM7Z
POL RNA
100%FS
No RNA
+ PAV6 RNA

CHAPTER 3. GENES AND *CIS*-ACTING SEQUENCES INVOLVED IN REPLICATION OF BARLEY YELLOW DWARF VIRUS-PAV RNA

A paper published in *Virology* **212**, 186-195 (1995)

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ABSTRACT

Deletion and point mutation analyses were employed to determine gene products and *cis*-acting signals involved in translation, replication and encapsidation of barley yellow dwarf virus (PAV serotype) RNA in oat protoplasts. Of the six open reading frames (ORFs), only ORFs 1 and 2, which include the putative RNA-dependent RNA polymerase gene, were required for replication. *In vitro* translation of these mutants revealed that sequence upstream of the shifty heptanucleotide was required for ribosomal frameshifting, and that a 3' translational enhancer stimulated translation more efficiently when located in closer proximity to the translated ORFs. Deletion of the coat protein gene reduced the accumulation of genomic but not subgenomic RNA. The carboxy-terminally extended form of the coat protein, produced by readthrough of its stop codon, was not required for encapsidation. Although the ORF6 product was not necessary, *cis*-acting

RNA signals in and around ORF6 were required for RNA replication. Defective RNAs harboring various deletions were not replicated in *trans* by the co-inoculated wildtype helper genome, suggesting that replication of PAV RNA may be coupled to translation.

INTRODUCTION

Barley yellow dwarf luteoviruses (BYDVs) have a positive sense RNA genome of ~5.7kb encoding at least six open reading frames (ORFs). Based on the genome organization, serological relationships and cytopathology, the luteoviruses have been divided into two subgroups (for reviews see Miller, 1994; Mayo and Ziegler-Graff, 1995; Miller *et al.*, 1995). Subgroup I includes the PAV and MAV serotypes of BYDV. Subgroup II includes the RPV serotype of BYDV, beet western yellows (BWYV), potato leaf roll (PLRV) viruses and others. The genomes of the two subgroups bear no resemblance to each other outside of the region that encodes the structural genes. Subgroup II luteoviruses encode a 5'-proximal ORF that is absent in subgroup I and lack a region homologous to the 3'-terminal 800 nucleotides (nt) of subgroup I genomes. The polymerase genes (ORF2) of subgroup I are most closely related to those of the diantho- and carmoviruses. In contrast, polymerases of subgroup II are similar to those of the sobemoviruses (Koonin and Dolja, 1993; Miller *et al.*, 1995). While deletion analysis has determined the necessity and roles of viral genes in replication of a subgroup II luteovirus (Reutenauer *et al.*, 1993), a thorough analysis has not been done on a subgroup I luteovirus. Although the effects of some mutations in a subgroup I luteovirus, BYDV-

PAV, have been reported (Young *et al.*, 1991; Filichkin *et al.*, 1994), some of the results presented here lead to different conclusions.

The subject of this paper, the PAV serotype of BYDV, contains six major ORFs (Fig. 1). ORFs 1 and 2, located in the 5' half of the genome, are translated directly from genomic RNA (Di *et al.*, 1993), while the 3' half is expressed from two subgenomic RNAs (sgRNAs) (Dinesh-Kumar *et al.*, 1992; Kelly *et al.*, 1994). A smaller, third sgRNA of unknown function was also identified (Kelly *et al.*, 1994). ORF2 is translated via a -1 ribosomal frameshift that occurs just before termination of translation of ORF1 (Brault and Miller, 1992), resulting in a 99 kDa fusion (P99) of the products of ORFs 1 and 2. The polymerase ORFs in BWYV (Veidt *et al.*, 1992) and PLRV (Prüfer *et al.*, 1992) genomes are also expressed by -1 frameshifting.

PAV ORF3 encodes the 22 kDa viral coat protein (CP). Contained within ORF3, but in a different reading frame, is ORF4 which encodes a 17 kDa protein (P17) (Fig. 1). ORF5 encodes a 50 kDa protein which is expressed as a 72 kDa fusion protein (P72) by translational readthrough of the ORF3 amber termination codon (Dinesh-Kumar *et al.*, 1992; Cheng *et al.*, 1994; Filichkin *et al.*, 1994; Wang *et al.*, 1995). Similar CP readthrough products have been described for other luteoviruses (Veidt *et al.*, 1988; Bahner *et al.*, 1990; Tacke *et al.*, 1990). The readthrough protein is essential for aphid transmission of BWYV (Brault *et al.*, 1995), but not for replication or encapsidation of BWYV (Reutenauer *et al.*, 1993) or PAV (Filichkin *et al.*, 1994) RNA .

ORF6 which has no known function can be translated from sgRNA2 *in vitro* (Kelly *et al.*, 1994). A frameshift mutation within ORF6 was reported to be incompatible with PAV RNA replication in protoplasts (Young *et al.*, 1991). In ten different PAV isolates, ORF 6 usually encodes a 4.3 kDa protein (Chaloub *et al.*, 1994), but in the infectious clone used in this study ORF6 encodes a 6.7 kDa protein which is larger due to a carboxy-terminal extension. A sequence in a region that ranges from the 3' end of ORF5 to the 5' half of ORF6 stimulates translation initiation at the ORF1 AUG from uncapped PAV genomic transcript by more than 30-fold *in vitro* (Wang and Miller, 1995).

In this paper, we have systematically introduced point mutations or deletions throughout the PAV genome in order to determine which viral gene products and *cis*-acting signals are required for RNA translation, replication and encapsidation in oat protoplasts. This work leads toward our goal of obtaining a complete understanding of gene function, expression and replication mechanisms of this widespread, economically important virus (D'Arcy and Burnett, 1995).

MATERIALS AND METHODS

Plasmid Construction

Maps of *in vitro* transcripts of all the plasmids used in this study are shown in Fig. 1. Transcripts are named for the plasmid from which they are transcribed with the lower case p omitted. Methods used to construct these plasmids are in Table 1. All plasmids were cloned in *Escherichia coli* strain DH5αf', except for those to be cut with *BclI* which

were grown in *dam*⁻ strain GM33. Base numbering refers to the PAV genome as in Miller *et al.*, (1988), with subscripts after restriction enzymes indicating the base after which the enzyme cleaves. Clones constructed by attempting to fill in the *Nde*I$_{4120}$ site instead turned out to have the two base sticky end deleted, as determined by sequencing.

Site-directed mutagenesis in construction of pPAV30 was done by the two step PCR method (Landt *et al.*, 1990). For both rounds of PCR, pPAV6 was used as template. The first round used the mutagenic oligomer: 5'-TTGGCCGATCGAGGATCTTC-3' (altered base is underlined) spanning bases 4913-4932 and the downstream primer: 5'-*GGGCCC*GGGTTGCCGAACTGCTCTTTCG-3', complementary to bases 5677-5656 with six nonviral bases at the 5' end (italics). The PCR product was gel-purified and used as downstream primer paired with the upstream primer: 5'-*CGCCATGG*TCATTACGATGAGTGTCAGT-3' (bases 3423-3442 with eight nonviral bases in italics). The resulting PCR product was digested with *Sca*I and *Xma*I, gel-purified and cloned into *Hpa*I-*Xma*I-cut pPAV6. (*Xma*I recognizes the same sequence as *Sma*I.) Sequences across mutated regions of plasmids pPAV19, pPAV20, pPAV22, pPAV25, pPAV30 were determined using an ABI Model 373A DNA sequencer.

In vitro transcription

All plasmids were linearized with *Sma*I prior to transcription. RNAs were transcribed with T7 RNA polymerase using either the Megascript kit (Ambion, Austin, TX) or the large-scale method described by Promega (Promega Notes, Vol 39, 1992).

Final RNA concentration was determined spectrophotometrically. ^{32}P-labeled antisense RNA probes were synthesized by *in vitro* transcription as described by Promega (Madison, WI) using $\alpha[^{32}P]$CTP. pPAV1-1 was linearized with *Eco*RI prior to transcription with SP6 RNA polymerase. Plasmid pSP10 was *Hin*dIII-linearized prior to transcription with T7 RNA polymerase.

Protoplast inoculation and RNA analysis

Protoplasts were isolated from *Avena sativa* cv Stout suspension culture (cell line S226 obtained from Howard Rines, USDA/ARS, University of Minnesota) as described previously (Dinesh-Kumar and Miller, 1993). Total RNA was isolated from inoculated protoplasts with a small-scale procedure that uses aurintricarboxylic acid as an RNase inhibitor (Wadsworth *et al.*, 1988; Dinesh-Kumar and Miller, 1993). The procedure used for Northern blot hybridization was a modification of the method described by Dinesh-Kumar *et al.*. (1992). RNA (5-10 μg) was separated by electrophoresis on denaturing 1% agarose gels, blotted onto nylon membrane (Gene Screen, DuPont) in 25mM phosphate buffer and fixed by UV crosslinking. Membranes were pre-hybridized at 65°C for at least 3 hrs in 50% formamide, 5X SSC, 20mM sodium phosphate (pH 6.5), 1% SDS and 0.2 mg/ml polyanetholesulfonic acid (Sigma). Hybridization was performed in the same buffer containing 500,000 cpm/ml of ^{32}P-labeled RNA transcripts for 12-16 hr at 65°C. Following hybridization, membranes were washed twice with 2X SSC/0.1% SDS for 3 min each at room temperature and then washed once with 0.1X SSC/0.1% SDS for 20 min

at 65° C. Blots were dried and exposed to X-ray film with an intensifying screen at -80°C.

Nuclease Protection Encapsidation Assay

RNA protected from cellular nucleases was used as a measure of encapsidation by the method of Reutenauer *et al.*, (1993). Pelleted, infected protoplasts were homogenized in 200 µl PIPES buffer and incubated for 0 (positive control) or 30 min at 37°C. Following incubation in cell lysates, nuclease-resistant RNA was isolated by the aurintricarboxylic acid method (above) and analyzed by Northern blot hybridization. Radioactive bands were detected and quantitated using a Phosphorimager 400E and Imagequant 3.3 software (Molecular Dynamics, Sunnyvale, CA). In the spike experiment, 500 ng of virions were added to uninoculated, lysed protoplasts, prior to the 0 (control) or 30 min incubation at 37°C. Virions were purified from infected plants by the method of Waterhouse *et al.*, (1986) modified as follows: infected tissue was powdered in liquid nitrogen prior to extraction, and cellulases were omitted from the process.

RESULTS

Effect of deletions on translation of ORFs 1 and 2

A series of deletions in ORFs 1 and 2 was produced to identify the roles of these ORFs in viral replication. The deletions within ORF1 (PAV19, PAV20 and PAV26 through PAV29) were designed not to change the reading frame because expression of

ORF2 requires translation of ORF1 followed by ribosomal frameshifting. To verify the effects of the deletions, transcripts from *Sma*I-linearized plasmids were translated *in vitro*. PAV26 and PAV27 transcripts gave the expected truncated pre-frameshift product of ORF1 (24 kDa) and the 84 kDa frameshift product (Fig. 2). The internally deleted product of ORF1 migrated more slowly than its expected size of 24 kDa, consistent with the anomalously slow migration of P39 (Di *et al.*, 1993). The shortened form of the ORF1 product from PAV28, (expected MW 7.9 kDa), is at the bottom of the gel in Fig. 2. The pre-frameshift products of PAV29 (4.7 kDa), PAV19 and PAV20 (both 2 kDa), if present, were too small to be detected. The presence of frameshift products of PAV28 and PAV29 (68 and 65 kDa, respectively) indicates that deletions up to nucleotide 1045 did not prevent frameshifting. However, the absence of frameshift products of PAV19 and PAV20 (≈62 kDa), which contain an in-frame deletion in ORF1 up to base 1115, suggests that sequences required for frameshifting have been deleted. Apparently, a region between bases 1045 and 1115, upstream of the shifty heptanucleotide (bases 1152-1158), is necessary for ribosomal frameshifting. PAV25 which has a full-length ORF1, but greatly deleted ORF2, missing bases beyond 1265, gave substantial amounts of the predicted 44.5 kDa frameshift product. Thus, no sequences between bases 1265 and 4120 are needed for frameshifting *in vitro*.

The *in vitro* translation products also revealed the effects of moving the recently described 3' translation enhancer (3'TE) (Wang and Miller, 1995) closer to the first ORF. This sequence, located between bases 4516 and 5009, is required for efficient translation

initiation of ORF1 in uncapped PAV transcripts (Wang and Miller, 1995). Transcripts with deletions that moved this sequence closer to ORF1 gave larger amounts of products of both ORF1 and the ORF1-2 frameshift fusion than the corresponding transcripts that lacked such deletions (Fig. 2, compare translation products of PAV26 with 27, and PAV25 with PAV6).

All deletions in ORFs 1 and 2 destroyed infectivity

To test their ability to replicate, the above transcripts were electroporated into oat protoplasts. Two other mutants were also tested: PAV34, in which the 3' two-thirds of ORF2 was deleted, and FLFSM4 in which one base (C) was inserted in the ORF1 stop codon, causing ORFs 1 and 2 to be fused into a large ORF that gives only the P99 translation product (Di *et al.*, 1993). After 48 hr, viral RNA replication products were detected by Northern blot hybridization. Wildtype (PAV6) RNA replicated efficiently, giving large amounts of genomic and three subgenomic RNAs with predicted sizes of about 3000, 800 and 300 nt (Dinesh-Kumar *et al.*, 1992; Kelly *et al.*, 1994) (Fig. 3A). Levels of all replicating RNAs in this study increased steadily over time in infected protoplasts (data not shown). No genomic, or subgenomic RNAs were detected in cells inoculated with any of the above mutants (Fig. 3A) (data not shown for FLFSM4 and PAV34). Very low molecular weight RNA was detected in all samples, which prevents us from concluding that small amounts of sgRNA3 were not produced, but we presume that

this smear is degraded inoculum. Thus, it is probable that ORFs 1 and 2 are required for virus replication.

Mutations and deletions in ORFs 3 and 4

All deletions in ORFs 3 and 4 removed parts of both ORFs because they overlap. To selectively knock out expression of the individual ORFs, site-directed mutagenesis was used to alter the start codons. The start codon of ORF4 was changed to GCG (PAV32) or ACG (PAV33). These mutations were designed so as not to modify the amino acid sequence of the overlapping ORF3 (Dinesh-Kumar and Miller, 1993). Initiation at the next downstream AUG in ORF4 would give rise to an N-terminally truncated 14 kDa product. PAV32 and PAV33 replicated, but accumulated less RNA than wildtype (Fig. 3B). However, cells that had been inoculated with PAV32 and PAV33 gave ELISA readings of A_{405} = 1.09 and 1.16, respectively, using antibody raised against virions. This is similar to A_{405} = 0.96 in PAV6-infected cells. Thus, the complete 17 kDa product of ORF4 is not essential for virus replication in protoplasts.

Transcripts containing the point mutation that eliminated the ORF3 (CP) start codon (PAV31) or deletions in ORFs 3, 4 and 5 (PAV23, PAV10 and PAV12) replicated, but yielded less genomic RNA than wildtype (Fig. 3B). The genomic RNAs and sgRNA1's of PAV23, PAV10 and PAV12 were smaller than wildtype as predicted by their 1334, 999 and 1727 base deletions, respectively. The subgenomic RNAs of PAV23 and PAV12 accumulated to high levels. As expected, the CP ORF mutants that were tested

(PAV31 and PAV10) gave ELISA readings (A_{405} = 0.02 and 0.04, respectively) similar to that obtained from uninoculated cells (A_{405} = 0.02). We conclude that replication can occur in the absence of coat protein but accumulation of genomic RNA is greatly reduced.

Effect of coat and readthrough proteins on PAV genomic RNA stability

We next investigated the role of CP in enhancing genomic RNA accumulation. Reduced genomic RNA could be due to one or both of the following reasons. *(i)* Cellular nucleases degrade nonencapsidated genomic RNA. (Subgenomic RNA is not encapsidated, so it would not be affected by absence of CP.) *(ii)* CP serves a specific regulatory role for high level synthesis of genomic but not subgenomic RNA. To test the role of CP in protecting genomic RNA, we performed a simple assay in which nucleases of lysed cells degrade nonencapsidated RNAs (Reutenauer *et al.*, 1993). Cells infected with various RNAs were lysed and incubated for 30 min at 37°C, prior to RNA extraction and Northern blot hybridization. In the control cells, RNA was extracted immediately upon cell lysis.

Initial experiments were designed to test how well encapsidated RNA resists the lysed cell treatment in order to estimate the fraction of total genomic RNA in infected cells that is encapsidated. In lysates of cells that had been infected with PAV6 or uncloned RNA from purified PAV virus particles, 2 to 10% of the genomic RNA survived the 37° incubation (Fig. 4). In general, none of the subgenomic RNAs survived lysate

treatment, supporting previous observations that they are not encapsidated (Kelly *et al.*, 1994).

It was possible that a substantial percentage of the progeny genomic RNA in protoplasts was not encapsidated, because the RNA was so sensitive to degradation in the lysate. To test this, lysed, uninfected protoplasts were spiked with 500 ng of virions from infected plants and then incubated at 37°. Only about 10% of the RNA in virions was protected (Fig. 4B). Within the experimental variation observed, this was not significantly different from the 2-10% resistance of the total genomic RNA in infected protoplasts. Thus, there may not be a large population of unencapsidated genomic RNA in infected cells 48 hr after inoculation, because the total genomic RNA in infected protoplasts is about as resistant as encapsidated virion RNA.

To test the roles of the coat protein and readthrough domain (ORFs 3 and 5), in conferring resistance to the cell lysate, cells were inoculated with PAV31, which does not synthesize coat protein and thus should not produce P72 via readthrough, or with PAV13 which has intact CP but a large deletion in the readthrough ORF. Progeny genomic RNA of PAV31 was completely degraded after 30 min in the cell lysate (Fig. 4A). However, because so little genomic RNA accumulated in these cells (0 min control), a small amount of lysate-resistant RNA, such as observed for wildtype, would not be detectable. In contrast, PAV13 progeny genomic RNA was clearly as resistant to lysate treatment as wildtype. The small amount of lysate-resistant sgRNA2 from PAV13-infected cells (Fig. 4A) is likely due to incomplete digestion of the very high levels of RNA that accumulated.

We conclude that ORF5 is unnecessary for viral replication or particle assembly. These results concur with previous observations of Reutenauer *et al.*, (1993) and Filichkin *et al.*, (1994) for BWYV and PAV, respectively, and contrast with the first report of Young *et al.*, (1991), in which frameshift mutations in ORF5 were lethal for virus accumulation in protoplasts.

Deletions in and around ORFs 5 and 6

PAV12, PAV10, PAV23 and PAV13, discussed above, contain deletions in ORF5 (Fig. 1) and were capable of replication. While PAV13 RNA, in which bases 3788-4515 were deleted, replicated to higher levels than wildtype, PAV22, in which the deletion extends from base 3788 to 4837, did not replicate (Fig. 3B). The lack of replication of PAV22 cannot be attributed to the absence of the carboxy-terminus of P72. The deletion in PAV13 introduces a frameshift, so it also does not express the carboxy terminus of P72. Thus, the lack of replication of PAV22 must be due to deletion of *cis*-acting signals required for virus replication.

The entire ORF6 plus flanking RNA was deleted in PAV24 (Fig. 1) which was unable to replicate (Fig. 3B). To determine whether this was due to the lack of the ORF6 product, or *cis*-acting signals, PAV30 was constructed. PAV30 is identical to PAV13 except that the start codon of ORF6 was altered to AUC. PAV30 replicated efficiently (Fig. 3B) and yielded high levels of viral antigen (A_{405} = 1.23 vs 1.22 for PAV13), but less progeny RNA than PAV13, which differs by only one base. sgRNA1 was reduced and

sgRNA2 was completely absent in cells infected with PAV30, but present in cells infected with PAV13. sgRNA3 was unaffected. Low levels of apparently wildtype-sized sgRNA1 appear unexpectedly in progeny RNAs of PAV13 and PAV30. When these inoculations were repeated several times, this RNA was not detected, but the relative levels of other sgRNAs were reproducible. The most striking result was that destroying the start codon of ORF6, which knocked out translation of the ORF (there are no other AUG codons), also prevented accumulation of sgRNA2 from which ORF6 is presumably translated (Kelly *et al.*, 1994). The fact that PAV30 but not PAV24 can replicate, indicates that the RNA sequence encoding or flanking ORF6 (between *Bam*HI4837 and *Bcl*I5190) is required for replication in oat protoplasts, but the protein product of ORF6 is not required. However, the reduced levels of PAV30 progeny compared to PAV13, indicate that the ORF6 product may play a minor supporting role in replication.

Lack of complementation of defective PAV transcripts by infectious transcripts

To test whether various deletions knocked out replication because of lack of a protein product (*trans*-acting factor), or because a *cis*-acting sequence in the RNA was disrupted, we attempted to replicate defective RNAs in the presence of wildtype (PAV6) transcripts in *trans*-complementation experiments. Such complementation has been demonstrated with other plant viruses (Rao and Hall, 1990; van der Kuyl *et al.*, 1991a; Ogawa *et al.*, 1991; White and Morris, 1994) but not with any luteovirus. In pairwise combinations, PAV6 was co-electroporated with eight of the defective transcripts

including PAV20, PAV22, PAV34, or PAV35 (Fig. 5) or PAV24 through PAV27 (data not shown). In the case of PAV27, several ratios of PAV6:defective RNA were used as inoculum, ranging from 20:1 to 1:20 (data not shown). None of the mutants was replicated by the co-inoculated helper genome (PAV6) under any conditions tested.

DISCUSSION

Expression and roles of ORFs 1 and 2

The P39 and P99 proteins are almost certainly essential, because all mutations in ORFs 1 and 2 knock out the ability of PAV RNA to replicate in oat protoplasts. The deletion mutants in ORF1 might suggest that a *cis*-acting signal within this ORF could be necessary, but the inability of FLFSM4 to replicate indicates that the P39 product itself is required. FLFSM4 has the complete ORF1 coding region, but expresses only P99 and not P39. The requirement for ORF1 contrasts with the report by Young *et al.*, (1991), in which a frameshift mutation near the 5' end of ORF1 did not destroy infectivity as measured by ELISA. However, our results concur with those of Reutenauer *et al.*, (1993) who found the pre-frameshift ORF of BWYV to be essential.

Many plant viruses express the polymerase via a translational fusion with an upstream ORF, either by in-frame readthrough or by frameshifting. In other viruses in which the polymerase gene was mutated so that it is in the same frame as the upstream ORF, infectivity was destroyed (Ishikawa *et al.*, 1986; Hacker *et al.*, 1992; Dalmay *et al.*, 1993). The function of these upstream ORFs, expressed both independently and fused

with a polymerase ORF, is unknown. Habili and Symons (1989) assert that many of these ORFs, including ORF1 of PAV have homology to helicases, although Koonin and Dolja (1993) dispute this. Because all mutants that prevent expression of full-length ORF2 have deletions in either ORF1 or ORF2, it is formally possible that the lack of replication of the ORF2 deletion mutants could be due to lack of *cis*-acting signals rather than the gene product itself. However, a requirement for the ORF2 gene product is highly likely, as polymerase genes are required for replication of all RNA viruses tested including BWYV (Reutenauer *et al.*, 1993), turnip crinkle virus (Hacker *et al.*, 1992) and cymbidium ringspot virus (CymRSV) (Dalmay *et al.*, 1993), which have homology to PAV.

Lack of frameshifting by PAV19 and PAV20 in which bases up to 1137 were deleted (Fig. 2) indicates that sequence upstream of the shifty site (bases 1152-1158) is required. This upstream region includes a large, stable stem-loop immediately adjacent to the 5' end of the shifty site in all subgroup I luteoviruses and related pea enation mosaic virus RNA2 (Miller *et al.*, 1995). Such a structure is not known to be present or required upstream of -1 frameshift sites of other viruses. Although our lab reported that the upstream stem-loop was not necessary for frameshifting by the out-of-context frameshift region *in vivo* (Brault and Miller, 1992), the amount of frameshifting by full-length RNA is greater (Di *et al.*, 1993) and may respond to different *cis*-acting signals (Wang and Miller, 1995).

Role of coat protein in genomic RNA accumulation

We used the method of Reutenauer *et al.*, (1993) who showed that no lysate-resistant RNA was produced by CP-lacking mutants of BWYV. Because we found that RNA known to be encapsidated was as sensitive to the lysate as total RNA in infected cells that express coat protein, the results are consistent with the possibility that most of the genomic RNA in PAV-infected cells is encapsidated and that the reduction in accumulation in cells inoculated with transcripts that cannot express coat protein is due entirely to lack of protection from cellular nucleases. However, we cannot rule out the possibility that CP also plays a more direct role in regulating genomic RNA synthesis. The finding that the protoplasts inoculated with virus having a defective CP gene accumulate less viral RNA than those inoculated with wild-type virus has been reported for several viruses (Sacher and Ahlquist, 1989; Allison *et al.*, 1990; Ishikawa *et al.*, 1991; Van Der Kuyl *et al.*, 1991b; Chapman *et al.*, 1992; Hacker *et al.*, 1992; Reutenauer *et al.*, 1993).

Effect of mutations in ORF4

Neither the sequence in ORF4, nor its protein product are required for replication of PAV RNA in protoplasts, as shown by efficient replication of mutants in which the entire ORF4 was deleted (PAV10, PAV12, and PAV23). It has been shown for PAV (Dinesh-Kumar and Miller, 1993) and PLRV (Tacke *et al.*, 1990) that the level of initiation at the ORF4 AUG exceeds that of the CP ORF. We also showed that translation

initiation at the CP AUG decreased upon deletion of the ORF4 AUG (Dinesh-Kumar and Miller, 1993). However, the same mutations in the ORF4 AUG, when present in infectious RNA (PAV32 and PAV33), did not affect CP levels based on ELISA. Thus the steady state levels of viral proteins in a virus infection are determined by more than simply the efficiency of translation initiation. It is possible that an N-terminally truncated form of ORF4 could be translated by leaky scanning initiation at the next in-frame AUG after the altered one in PAV32 and PAV34. This would generate a 14 kDa product which has been detected by *in vitro* translation (C.M. Brown, personal communication), but not in PAV32-infected protoplasts (S.M. Gray, personal communication).

Previously, we and others speculated that ORF4 may encode a 5' genome-linked protein (VPg) (Miller *et al.*, 1988; Keese and Gibbs, 1992). VPg's are likely to be essential for RNA replication. However, the dispensibility of the ORF4 for replication shown here, suggests that it does not encode a VPg. Furthermore, no VPg has been detected on PAV RNA (L. Domier, personal communication). Thus, VPg's may be present on subgroup II but not subgroup I luteoviral genomic RNAs (Miller *et al.*, 1995).

Roles of essential *cis*-acting signals in and around ORFs 5 and 6

The effects of mutations from the 3' portion of ORF5 to beyond the 3' end of ORF6 indicated that the proteins encoded were unnecessary, but a *cis*-acting signal was essential for RNA replication in protoplasts. This is consistent with a requirement for the *cis*-acting sequence (3'TE, Fig. 1) between the *Sca*I4515 and *Pst*I5009 sites, that mediates

efficient translation of ORF1 from uncapped genomic RNA in wheat germ extracts (Wang and Miller, 1995). The role of a cap-independent translation signal is unclear, as the nature of the 5' end of PAV genomic RNA is unknown. However, capping of PAV6 transcripts greatly reduced infectivity (Dinesh-Kumar, 1993). Sequence adjacent to the 3'TE also affects translation (Wang and Miller, 1995). This may explain why Young *et al.*, (1991), found that a frameshift mutation at the *Pst*I site in ORF6 was lethal for replication, but our single base change to knock out the AUG (PAV30) was not lethal (Fig. 3B). The complete absence of sgRNA2 in PAV30-infected protoplasts was surprising. This suggests that either G4922 is essential for the subgenomic promoter function despite being 113 bases downstream of the sgRNA2 5' end (Kelly *et al.*, 1994), or translation of sgRNA2 is required for its stability.

No homologs to ORF6 or the flanking *cis*-acting sequences are apparent in BWYV and other subgroup II luteovirus genomes (Miller *et al.*, 1995). ORF6 is lacking, but some nucleotide sequences homologous to PAV are present, in the 3' end of subgroup I-like soybean dwarf luteovirus RNA (Rathjen *et al.*, 1994). A similar small ORF is present in the 3' ends of tombusviral genomes (Boyko and Karasev, 1992). Dalmay *et al.* (1993) observed that altering only the AUG of this ORF in CymRSV did not affect replication in protoplasts or plants, but that deletions in and immediately upstream of the ORF eliminated replication. Thus tombusviruses, which have homology to subgroup I luteoviruses in the polymerase gene (Koonin and Dolja, 1993) but not in the small 3' ORF,

may resemble PAV by having essential *cis*-acting signals in and around the small 3' ORF whose protein product is not essential.

Cis-preferential replication?

We consider three explanations for lack of *trans*-complementation of replication-defective genomes by infectious genomes. *(i)* Protoplasts could not be co-electroporated with two separate RNAs in our conditions. This is unlikely because the synergy observed in mixed infections of PAV and RPV RNAs, and the ability of satellite RNA to be supported by BYDV genomic RNA in protoplasts demonstrates that co-transfection with multiple RNAs occurs readily (Silver *et al.*, 1994). *(ii)* All of the eight deletion mutants tested lack *cis*-acting signals required for RNA replication. Based on the different deletions used, this would require that many such signals exist all across the genome, unlike any known virus. *(iii)* PAV RNA replication may be *cis*-preferential, i.e. the replicase may copy only the RNA from which it was translated. Coupling between genome translation and replication has also been reported for poliovirus (Novak and Kirkegaard, 1994) and several plant viruses (White *et al.*, 1992; van Bokhoven *et al.*, 1993; Weiland and Dreher, 1993). However, the impossibility of *trans*-replication cannot be assured because certain combinations of deleted RNAs may be able to overcome this *cis*-limitation (Weiland and Dreher, 1993; Novak and Kirkegaard, 1994). Additional studies are needed to identify and characterize further the *cis* and *trans* acting signals essential for PAV RNA replication.

ACKNOWLEDGMENTS

The authors thank Cindy Paul and Holly Sipes for construction of clone pPAV34, Shanping Wang for clone pPAV35, and Randy Beckett for virus preparation. This work was funded by USDA Biotechnology Risk Assessment Grant no. 94392100531 and USDA National Research Initiative Grant no. 9137036424. This is paper no. J-16377 of the Iowa State University Agricultural and Home Economics Experiment Station.

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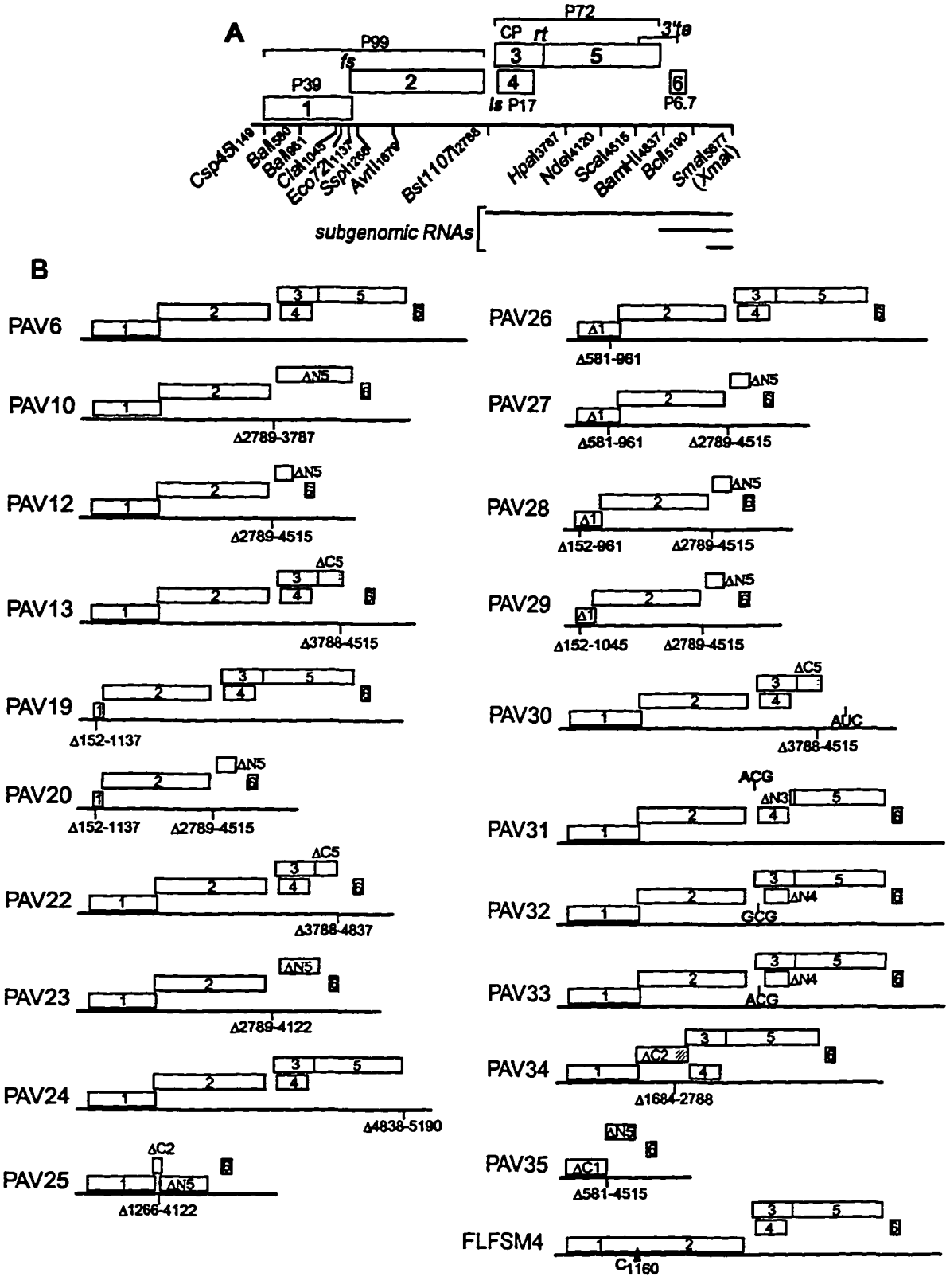
Table 1. Construction of plasmids used in this study

Plasmid	Deleted or altered bases	Construction
pPAV6	None (wildtype)	Di et al., (1993)
pPAV10	2789-3787	Cut pPAV6 with Bstl107I$_{2789}$ and HpaI$_{3787}$, fill in, religate.
pPAV12	2789-4515	Insert ScaI$_{4515}$-XmaI$_{5677}$ fragment from pPAV6 into pPAV6 cut with Bstl107I$_{2788}$ and XmaI$_{5677}$.
pPAV13	3788-4515	Insert ScaI$_{4515}$-XmaI$_{5677}$ fragment from pPAV6 into HpaI$_{3787}$-XmaI$_{5677}$-cut pPAV6.
pPAV19	152-1137	Cut pPAV6 with Csp45I$_{149}$, fill in, cut with Eco72I$_{1137}$, religate.
pPAV20	152-1137, 2789-4515	Cut pPAV12 with Csp45I$_{149}$, fill in, cut with Eco72I$_{1137}$, religate.
pPAV22	3788-4837	Insert filled-in BamHI$_{4837}$-XmaI$_{5677}$ fragment of pPAV6 into HpaI$_{3787}$-XmaI$_{5677}$-cut pPAV6.
pPAV23	2789-4122	Cut pPAV6 with Bstl107I$_{2789}$ and NdeI$_{4120}$, fill in, religate.
pPAV24	4838-5190	Cut pPAV6 with BamHI and BclI$_{5190}$. Gel-purify and ligate two largest (3097 nt and 4967 nt) fragments.
pPAV25	1266-4122	Cut pPAV6 with Ssp$_{1263}$ and NdeI$_{4120}$, fill in, religate.
pPAV26	581-961	Cut pPAV6 with BalI and religate.
pPAV27	581-961, 2789-4515	Cut pPAV26 with Bstl107I$_{2788}$ and ScaI$_{4515}$, religate
pPAV28	152-961, 2789-4515	Cut pPAV12 with Csp45I$_{149}$ and BalI, fill in, religate.
pPAV29	152-1045, 2789-4515	Cut pPAV12 with Csp45I$_{149}$ and ClaI$_{1045}$, fill in, religate.
pPAV30	3788-4515 and G$_{4922}$→C (Alters ORF6 start codon to AUC)	Site-directed mutagenesis as described in Methods.
pPAV31	U$_{2859}$→C (Alters CP ORF start codon to ACG)	Clone Bstl107I$_{2788}$-SalI$_{2985}$ fragment of pM5 (Dinesh-Kumar and Miller, 1993) into Bstl107I$_{2788}$-SalI$_{2985}$ cut PAV6.
pPAV32	A$_{2901}$→G, U$_{2902}$→C (Alters ORF4 start codon to GCG)	Clone Bstl107I$_{2788}$-SalI$_{2985}$ fragment of pM7 (Dinesh-Kumar and Miller, 1993) into Bstl107I$_{2788}$-SalI$_{2985}$ cut PAV6.
pPAV33	U$_{2902}$→C (Alters ORF4 start codon to ACG)	Clone Bstl107I$_{2788}$-SalI$_{2985}$ fragment of pM9 (Dinesh-Kumar and Miller, 1993) into Bstl107I$_{2788}$-SalI$_{2985}$ cut PAV6.
pPAV34	1684-2788	Cut pPAV6 with AvrII$_{1679}$, fill-in, cut with Bstl107I$_{2788}$, religate.
pPAV35	581-4515	Cut pPAV6 with BalI$_{581}$ and ScaI$_{4515}$, religate.
pSP10	Antisense to bases 4228-5677. Probe for Northern blot.	(Dinesh-Kumar et al., 1992)
pPAV1-1	Antisense to bases 1-546. Probe for Northern blot.	Clone SacI-XbaI fragment of pPAV1 (Silver et al., 1994) into SacI-XbaI cut pGEM3Zf(+).
pFLFSM4	U$_{1159}$AG→U$_{1159}$CAG Fuses ORFs 1 & 2 by disrupting ORF1 stop.	Di et al., (1993)

FIG. 1. A. Genome organization of PAV. Open reading frames are indicated by number inside the box and by size of protein product outside the box. Sites of translational events are abbreviated as follows: *fs*=frameshift, *ls*=leaky scanning, *rt*=readthrough, *3'te*=3' translation enhancer. Bold lines below ORFs indicate genomic (with relevant restriction sites) and subgenomic RNAs. **B.** Organizations of deletion and point mutants of the genomic transcripts. PAV6 is the infectious, wild-type transcript. Site of deletion and range of bases deleted are indicated by Δ followed by base positions under short vertical line. ΔN or ΔC followed by ORF number indicates whether N or C terminus, respectively, of the ORF was deleted. Absence of N or C in deleted ORF indicates internal, in-frame deletion. Shaded regions indicate change in amino acid sequence encoded by ORF due to frameshift mutation caused by the deletion. Altered start codons are indicated by the new codon at the appropriate position in mutants PAV30 through PAV33, and the remainder of the ORF beginning at the next in-frame AUG is shown.


A
P72
P99
CP
rt
3'te
fs
3
5
P39
2
4
6
1
ls P17
P6.7
Csp45I149
BalI580
BalI961
ClaI1045
Eco72I1137
SspI1266
AvrII1679
Bst1107I2788
HpaI3787
NdeI4120
ScaI4515
BamHI4837
BclI5190
SmaI5877 (XmaI)
subgenomic RNAs
B
PAV6
PAV10
Δ2789-3787
PAV12
Δ2789-4515
PAV13
Δ3788-4515
PAV19
Δ152-1137
PAV20
Δ152-1137
Δ2789-4515
PAV22
Δ3788-4837
PAV23
Δ2789-4122
PAV24
Δ4838-5190
PAV25
Δ1266-4122
PAV26
Δ581-961
PAV27
Δ581-961
Δ2789-4515
PAV28
Δ152-961
Δ2789-4515
PAV29
Δ152-1045
Δ2789-4515
PAV30
AUC
Δ3788-4515
PAV31
ACG
PAV32
GCG
PAV33
ACG
PAV34
Δ1684-2788
PAV35
Δ581-4515
FLFSM4
C1160

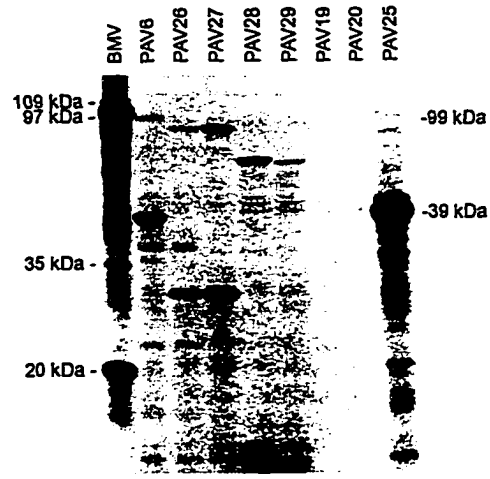


FIG. 2. *In vitro* translation of PAV transcripts. Equimolar amounts (0.1 pmol) of uncapped T7 transcripts (indicated at top of each lane) were translated in wheat germ extracts (Promega), and translation products analyzed as described previously (Di *et al.*, 1993). Sizes of brome mosaic virus (BMV) RNA translation products are indicated at left. Mobilities of PAV6 P39 (39 kDa) and frameshift (99 kDa) products are indicated at right.

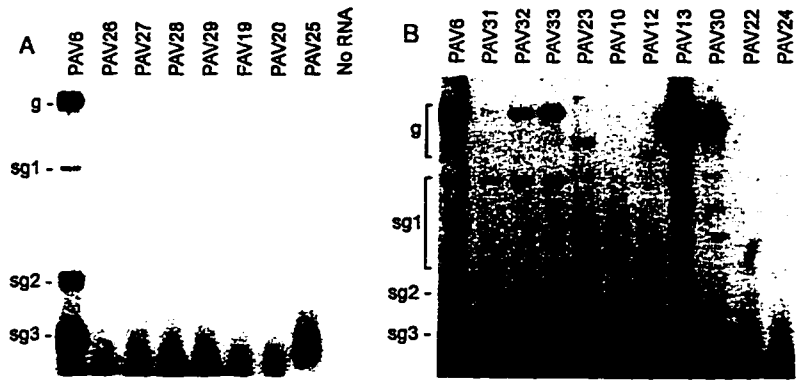


FIG. 3. Northern blot hybridization of RNA from cells inoculated with mutant PAV transcripts. Total RNA was exracted from protoplasts 48 hr after inoculation with the transcripts indicated above each lane. Blots were probed with transcript from pSP10, complementary to the 3'-terminal 1450 bases of the PAV genome. ***A.*** Products of mutants with deletions in ORFs 1 or 2. No RNA indicates total RNA from uninoculated protoplasts. Mobilities of genomic (g) and subgenomic (sg) RNAs 1, 2 and 3 from cells infected with PAV6 (wildtype) transcript are at left. ***B.*** Products of transcripts with mutations in the 3' half of the genome. Brackets span size range of deletion-containing genomic and sgRNA1.

FIG. 4. Northern blot hybridization of RNAs exposed to cell lysate. Protoplasts were lysed and incubated for 0 min (C) or 30 (T) min prior to RNA extraction. ***A.*** Total RNA was isolated from protoplasts 48 hr after inoculation with the transcripts indicated above each lane. RNAs were blotted and probed with 3'-specific complementary RNA as in Fig. 3. Duplicate samples are under brackets. ***B.*** Cell lysate-treated RNAs in infected cells or from purified virions. Total RNA was isolated from protoplasts 48 hr after inoculation with uncloned RNA purified from virions in lanes marked "Infected". "Spiked" indicates RNA extracted from cell lysates of uninonculated protoplasts to which 500 ng of virions had been added and incubated for 0 (C) or 30 (T) min, prior to extraction.

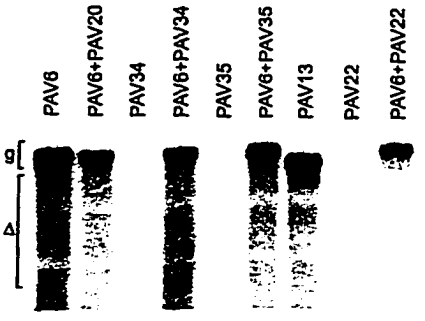


FIG. 5. Northern blot hybridization of RNA from cells inoculated with mixtures of mutant and wildtype PAV transcripts. RNA was extracted from protoplasts 48 hr after inoculation with 5 pmol (3-10 μg, depending on size of deletion) of each transcript indicated above each lane. Blots were probed with 5'-complementary transcript from pPAV1-1 which is incapable of detecting subgenomic RNAs. g, size range of functional, replicating genomes. Δ, size range of expected progeny of defective genomes.

CHAPTER 4. MUTATIONAL ANALYSIS OF THE PROMOTER FOR THE BARLEY YELLOW DWARF LUTEOVIRUS 3.0-kb SUBGENOMIC RNA

A paper to be submitted to Virology

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ABSTRACT

The synthesis of subgenomic mRNAs (sgRNAs) is an important strategy by which the internally located open reading frames (ORFs) are expressed and regulated during replication. Barley yellow dwarf luteovirus (BYDV)-PAV RNA has three sgRNAs. Mutant BYDV genomic transcripts were used to identify sequences required for sgRNA synthesis and virus replication in oat protoplasts. Time course experiments indicate that the accumulation of the sgRNAs parallels the accumulation of the genomic RNA. Mutation analysis was used to map BYDV-PAV sgRNA1 promoter because subgenomic promoters are putative hotspots for viral recombination. Using site-directed mutagenesis, we have shown that the sgRNA1 transcription start site is at base 2670. Altering the initiation base at 2670 drastically reduced both the genomic RNA and sgRNA1 accumulation in oat protoplasts. Results of mutagenesis also indicate that the nucleotides

immediately surrounding the sgRNA1 start site regulate subgenomic promoter activity. Mutating a more distant but conserved ACAAA domain, a candidate promoter sequence, decreased both the genomic RNA and sgRNA1 accumulation. Computer-generated secondary structures for the putative sgRNA1 promoter of several members of subgroup I luteovirus revealed a conserved stem-loop structure near the transcription start site of sgRNA1. In this study, we also show that the amino acids encoded by the 3'-terminal 90 bases of the putative polymerase gene (ORF2) are essential for viral replication in protoplasts.

INTRODUCTION

Genes in the 3' regions of many plant viral genomes are expressed via subgenomic RNAs (sgRNAs). Subgenomic RNAs are plus (+) strand, 3' coterminal copies of the genomic RNA, truncated at the 5' end to allow for ribosomal access to each of the internal genes. Three mechanisms for the synthesis of sgRNAs have been proposed: the first, discontinuous leader RNA-primed transcription, is shown to occur during the production of coronavirus sgRNAs (Lai *et al.*, 1984; Lai *et al.*, 1990). The second mechanism involves leader recombination during minus-strand synthesis of coronavirus (Chang *et al.*, 1996). In this mechanism, polymerase jumps from the sgRNA start site on plus-strand during the minus-strand synthesis and re-initiates near the 5' end of the genome. The third mechanism, internal initiation of transcription by the viral replicase, on minus (-) strand RNA template has been shown to occur *in vitro* for brome mosaic virus (BMV; Miller *et al.*, 1985) and alfalfa mosaic virus (AlMV; van der Kuyl *et al.*, 1990) and *in vivo* for

turnip yellow mosaic virus (Gargouri *et al.*, 1989). Internal initiation is directed by specific subgenomic promoter sequences on the (-) strand.

Barley yellow dwarf luteovirus (BYDV) is the most widespread and economically important virus of small grain cereals, worldwide (D'Arcy and Burnett, 1995). Luteoviruses have been divided into two subgroups (for reviews see Miller *et al.*, 1995; Mayo and Zeigler-Graff, 1996). Subgroup I includes the PAV and MAV serotypes of BYDV. Subgroup II includes the RPV serotype of BYDV, potato leafroll virus (PLRV), beet western yellows virus (BWYV), and others. The polymerase genes of subgroup I are most closely related to those of the diantho- and carmoviruses. In contrast, polymerases of subgroup II are similar to those of sobemoviruses (Koonin and Dolja, 1993; Miller *et al.*, 1995).

The PAV serotype of BYDV, the subject of this paper, has a positive-sense RNA genome of ~5.7Kb encoding at least six open reading frames (ORFs; Fig. 1). Several gene expression strategies are used by BYDV-PAV and other luteoviruses (Fig. 1). These strategies include the transcription of sgRNAs (Dinesh-Kumar *et al.*, 1992; Kelly *et al.*, 1994), readthrough of an amber termination stop codon (Tacke *et al.*, 1990), translational frameshifting (Brault and Miller, 1992), the expression of overlapping, out-of-frame ORFs (Dinesh-Kumar and Miller, 1993) and cap-independent translation (Wang and Miller, 1995). The 5' ORFs of BYDV-PAV are translated directly from the genomic RNA (Di *et al.*, 1993) and have been shown to be essential for viral replication (Mohan *et al.*, 1995). The 3' half of BYDV-PAV genome is expressed from two sgRNAs (Dinesh-Kumar *et al.*,

1992; Kelly *et al.*, 1994). A smaller, third sgRNA of unknown function was also identified (Kelly *et al.*, 1994).

The subgenomic promoter regions on the (-) strand template responsible for directing the synthesis of sgRNAs have been well studied in few members of alphaviruses such as sindbis virus (Levis *et al.*, 1990). Among plant viruses, studies of *cis*-acting elements involved in sgRNA synthesis have been performed with BMV (French and Ahlquist, 1988), AlMV (van der Kuyl *et al.*, 1990; van der Vossen *et al.*, 1995), beet necrotic yellow vein virus (BNYVV; Balmori *et al.*, 1993), cucumber mosaic virus (CMV; Boccard and Baulcombe, 1993), cucumber necrosis tombusvirus (CNV; Johnston and Rochon, 1995), red clover necrotic mosaic virus (RCNMV; Zavriev *et al.*, 1996), potato virus X potexvirus (PVX; Kim and Hemenway, 1996 and 1997) and turnip crinkle virus (TCV; Wang and Simon, 1997). However, the subgenomic promoters of luteoviruses have not been characterized. The transcription start site of sgRNA1 of BYDV-PAV was originally identified as base 2769 (Dinesh-Kumar *et al.*, 1992) but then found to be base 2670 (Kelly *et al.*, 1994; G.Koev, personal communication). The correct start site can be identified by mutating these two start sites and then the sgRNA1 promoter can be mapped.

Mapping the subgenomic RNA promoter is important because subgenomic promoters are putative hotspots for viral recombination (Miller *et al.*, 1995). The boundaries where homology between the two luteovirus subgroups begins and ends occur at subgenomic RNA start sites. According to the recombination model for the origin of luteoviruses (Miller *et al.*, 1995); in a mixed infection, polymerase in the process of

synthesizing plus-strand of a related supergroup II virus could jump to the subgenomic promoter of a member of one luteovirus subgroup to generate a recombinant with the genome organization of the other luteovirus subgroup.

In this paper, we have used site-directed mutagenesis to investigate the importance of nucleotides (nt) immediately flanking the sgRNA1 start site. In addition, we have also identified the role of the conserved ACAAA motif in viral replication. This study will further our understanding of transcriptional control, viral replication and gene expression of BYDV.

MATERIALS AND METHODS

Plasmid Construction

DNA manipulations were performed as described in Sambrook *et al.* (1989). All plasmids were cloned in *Escherichia coli* strain DH5αf'. Base numbering refers to the PAV genome as in Miller *et al.* (1988). All mutations used in this study were generated by the two-step polymerase chain reaction (PCR) mutagenesis method of Landt *et al.* (1990). For both rounds of PCR in all the mutations, pPAV6 was used as the template. Transcripts were named for the plasmid from which they are transcribed with the lowercase "p" omitted.

(i) pPAV36: In mutant plasmid, pPAV36, a premature UAA stop codon was introduced in ORF2 at base 2650 by deleting a G at base 2641. The first round of PCR used the upstream mutagenic primer MB1298 (5'-

ACGACGTCTTACGG**TAA***GGGCCC*AACTCC-3') (stop codon in bold), containing an ApaI site (in italics) and spanning bases 2635-2664, and the downstream primer AM092401 (5'-TTGCGCGTCTAGGTCCTCTA-3'), complementary to bases 2894-2875. Details of the second round PCR and cloning are given below.

(ii) pKel6 and pKelf: Plasmids, pKel6 and pKelf were created to mutate the nucleotides immediately flanking the sgRNA1 start site without altering the initiation base (G at 2670). The first round PCR used the upstream mutagenic primer MB0388 (5'-GCCCAACTCCAG**TC[G/C/A]**GT[**T/C**]AAAGTGACGACTCCACAT-3')(altered bases in bold), spanning bases 2655-2690, and the downstream primer SPDK4 (5'-CTGAATTCGTTCACCACC-3'), complementary to bases 2867-2850. Kel6 mutation changed one amino acid (Val to Asp) whereas, Kelf maintained the same amino acid sequence as the wild-type (wt) PAV6.

(iii) pACAAA2: pACAAA2 was created to mutate the conserved ACAAA domain between bases 2729-2733, without changing the amino acid sequence. The upstream mutagenic primer MB1466 (5'-ACCGGAAAACCAC**AG[T/C]**CA**G**AACGAATATTAATTACC-3') (altered bases in bold), spanning bases 2714-2749 and the downstream primer SPDK3 (5'-CTTGTGTCATTTAGATTTGCGCGT-3'), complementary to bases 2910-2888, were used for the first round PCR.

(iv) p2670M: p2670M was constructed to mutate the sgRNA1 start site at base 2670. G at base 2670 was mutated to C, altering the amino acid, from Valine to Leucine. The

upstream mutagenic primer MB1467 (5'-CCAGAGTCTGAAGGTGACGACT-3') (altered base in bold), spanning bases 2663-2684, and the downstream primer SPDK3 (as above), were used for the first round PCR.

The second round PCR and cloning for all of the above mutations were done as follows: The product from the first PCR was gel-purified and used as downstream primer along with the upstream primer RB1100 (5'-TGGCTCTTGCACTTGAAC-3'), spanning bases 1927-1945, for the second round PCR. The resulting PCR product was digested with Bstl1071 and Tth111, gel-purified and cloned into Tth111-Bstl1071-cut pPAV6. Sequences across mutated regions of plasmids pPAV36, pKel6, pKelf, pACAAA2 and p2670M were determined using an ABI Model 373A DNA sequencer.

In Vitro transcription

All plasmids were linearized with SmaI prior to transcription. RNAs were transcribed with T7 RNA polymerase using either the Megascript kit (Ambion, Austin, TX) or the large-scale method described by Promega (Promega Notes, Vol. 39, 1992). Final RNA concentration was determined spectrophotometrically. ^{32}P-labeled antisense RNA probes were synthesized by *in vitro* transcription as described by Promega (Madison, WI), using [α-^{32}P]-CTP. Plasmid pSP10 was linearized with HindIII prior to transcription with T7 RNA polymerase. pSP9 was linearized with EcoRICRI prior to transcription with SP6 RNA polymerase.

Protoplast inoculation and RNA extraction

Protoplasts were isolated from *Avena sativa* cv stout suspension culture (cell line S226 obtained from Howard Rines, USDA/ARS, University of Minnesota) as described previously (Dinesh-Kumar and Miller, 1993). Total RNA was isolated from inoculated protoplasts either with a small-scale procedure that uses aurintricarboxylic acid as an RNase inhibitor (Wadsworth *et al.*, 1988; Dinesh-Kumar and Miller, 1993) or using the RNeasy RNA extraction kit (QIAGEN, CA).

Northern blot analysis

RNA was electrophoresed on a 1% denaturing agarose gel containing formaldehyde, blotted to nylon membrane (Gene Screen, DuPont) and hybridized with ^{32}P-labeled antisense RNA probe as described in Mohan *et al.* (1995). Blots were dried and exposed to X-ray film with an intensifying screen at -80°.

ELISA

ELISA plates were coated with PAV-specific polyclonal antibodies for three hours at 37° and blocked with 1% fat-free carnation dry milk solution. Plates were incubated with antigen (protoplast extract) overnight at 4°. The following day plates were washed three times (three minutes each), with phosphate-buffered saline containing 0.1% tween. Alkaline phosphatase-PAV polyclonal antibody conjugate was loaded onto the plates and

incubated for three hours at 37°. Plates were washed as above, and the substrate was added to the plates. ELISA readings (A_{405}) were taken using Dynatech minireader.

Computer analysis

Computer-predicted secondary structures were obtained using MFOLD program. The drawings of the stem-loop structures were drawn using loopDloop program

RESULTS

Time course of BYDV-PAV genomic and subgenomic RNA accumulation in oat protoplasts

Infection of oat protoplasts with wild-type transcripts synthesized from a full-length BYDV-PAV cDNA clone (pPAV6; Di *et al.*, 1993) resulted in accumulation of genomic RNA and three sgRNAs (Dinesh-Kumar *et al.*, 1992; Kelly *et al.*, 1994). The rate of accumulation and relative abundance of these RNAs in oat protoplasts at various times postinoculation (PI) were examined by northern blot analysis using a 3' PAV-specific probe. No genomic or sgRNAs were detected until 12 hr PI and RNA levels increased up to 48 hr PI (Fig. 2A and 2B). Similar observations have been reported for plant viruses such as cucumber necrosis virus (CNV; Johnston and Rochon, 1995), turnip crinkle virus (TCV; Wang and Simon, 1997) and potato virus potexvirus (PVX; Kim and Hemenway, 1997). Minus-strand genomic and sgRNAs were detected by 18 hr PI and the RNA levels increased up to 48 hr PI (data not shown). Accumulation of BYDV-PAV sgRNAs

paralleled that of the genomic RNA. As observed previously (Kelly *et al.*, 1994), sgRNA3 and sgRNA2 were transcribed in slightly larger amounts than sgRNA2 and sgRNA1, respectively. A slight decrease in the amount of genomic and sgRNAs at 72 hr PI was due, at least partially, to a decline in the number of viable protoplasts during the long period of incubation. A similar observation has been made for RNA accumulation of citrus tristeza virus in tobacco protoplasts (Navas-Castillo *et al.*, 1997). Although we did not test for cell viability, visual observation of protoplasts indicated slightly more number of dead cells at 72 hr PI compared to earlier time points. Graphic representation of genomic RNA and sgRNA1 accumulation and their ratios at different time points is shown in Figure 3A and 3B.

A premature stop codon 90 bases from the 3' end of the polymerase gene abolishes viral replication

Deletion and mutation analyses have been used to map the subgenomic promoters of BMV (Marsh *et al.*, 1988; French and Ahlquist, 1988; Smirnyagina *et al.*, 1994), BNYVV (Balmori et al., 1993), CNV (Johnston and Rochon, 1995) and TCV (Wang and Simon, 1997). To use this approach with BYDV-PAV would require introducing deletions in the 3'-end of ORF2, as the sgRNA1 start site (base 2670) is within the 3' end of the polymerase gene. In our previous study (Mohan *et al.*, 1995), we have shown that the polymerase gene is essential for viral replication in protoplasts. To identify whether deletions can be constructed in the 3' region of the polymerase gene without abolishing

viral replication, we introduced a premature UAA stop codon at base 2650 in the polymerase gene by site-directed mutagenesis. Wild-type (wt) PAV6 and PAV36 (mutant with premature stop codon) transcripts were electroporated into oat protoplasts. After 48 hours, viral RNA replication products were detected by northern blot hybridization.

PAV6 (wt) RNA replicated efficiently, giving large amounts of genomic (5.7kb) and three sgRNAs with predicted sizes of about 3.0, 0.86 and 0.32kb (Fig. 4). No genomic, or sgRNA replication were detected in cells inoculated with mutant PAV36 transcript (Fig. 4). These results were confirmed by ELISA, in which the wt PAV6 RNA gave high ELISA readings (A_{405} = 1.38) whereas, the mutant PAV36 gave ELISA readings (A_{405} = 0.01) similar to that of the background (A_{405} = 0.01). Thus, the 3' terminal 90 bases of the polymerase gene (ORF2) are essential for BYDV-PAV replication in oat protoplasts.

Mutations near the sgRNA1 start site (base 2670)

We expect that bases at or near the start site (base 2670) are part of the promoter and necessary for sgRNA synthesis. To test this, PCR-based site-directed mutagenesis was used to mutate the nuleotides immediately flanking the start site at 2670. PAV6 sequence from base 2667-2675 has been altered from AGUGUGAAG (wt) to UCGGAUAAG in Kel6 mutant and to UCCGUUAAA in Kelf mutant (altered bases underlined and initiation base G is in bold) (Fig. 5A). In Kel6 mutant, 5 bases and 1 amino acid (Val to Asp) were altered, whereas, in Kelf mutant, 5 bases were altered without any

change in amino acid sequence (Fig. 5A). Kel6 and Kelf mutant transcripts replicated in oat protoplasts (5B). They produced only slightly reduced amounts of genomic RNA similar but very little or no sgRNA1 (Fig. 5B; compare lanes 3, 4, 5 and 6 with 1 and 2 with respect to sgRNA1). A faint band that migrates slightly slower than sgRNA1 is visible in all lanes that contain genomic RNA. This is an artefact caused by the front of the very high levels of ribosomal RNA displacing degraded genomic RNA. From these results we conclude that the nucleotides immediately flanking the sgRNA1 start site at base 2670 are essential for sgRNA1 accumulation in protoplasts.

Altering the base at which sgRNA1 synthesis initiates (G at 2670) drastically reduces both the genomic RNA and sgRNA1 accumulation

To determine the significance of the initiation base, we mutated the G at 2670 to C. This caused an unavoidable change in amino acid from valine (in wt) to leucine (in 2670M mutant). This mutant 2670M, was tested for its ability to accumulate sgRNA1 in oat protoplasts. sgRNA1 accumulation was drastically reduced in 2670M (Fig. 5B; lanes 9, 10 and 12). This mutation at 2670 also reduced the genomic RNA accumulation (Fig. 5B). In longer exposures, low amounts of genomic RNA was seen in the lanes of 2670M (data not shown). These results suggest that G at base 2670 is essential for genomic and sgRNA1 accumulation.

Mutating the conserved ACAAA domain reduces viral replication

A conserved ACAAA motif is found at or near the 5` ends of the genomic RNA and sgRNAs of several members of the luteovirus group (Fig. 6; Miller *et al.*, 1995) and selected related plant viruses such as southern bean mosaic sobemovirus-C strain (SBMV-C; Wu *et al.*, 1987), SBMV-B strain (SBMV-B; Othman and Hull, 1995), rice yellow mottle sobemovirus (RYMV; Yassi *et al.*, 1994), sweet clover necrotic mosaic dianthovirus RNA1 (SCNMV1; Ge *et al.*, 1993) and carnation ringspot dianthovirus RNA1 (CRSV1; Ryabov *et al.*, 1994). The ACAAA sequence occurs 16 times in the BYDV-PAV genome. One such ACAAA sequence is found between bases 2729-2733, 59 bases downstream of the sgRNA start site (2670). This ACAAA sequence was mutated to create the mutant ACAAA2. ACAAA sequence was altered to CCAGA (altered bases underlined), in addition to the two immediate upstream bases which were changed from UC (wt PAV) to AG in the mutant. The two upstream bases were mutated as they are conserved in many luteoviruses. In infected protoplasts, the genomic RNA and sgRNA1 accumulation was reduced by more than 50% (Fig. 5B; lanes 7, 8 and 9) for mutant ACAAA2 as compared to the wt PAV6 RNA accumulation (Fig. 5B; lanes 1 and 2). Decreased replication by mutant ACAAA2 indicates that the conserved ACAAA motif is essential for viral replication and accumulation of genomic RNA and sgRNA1 in protoplasts. All the mutants used in this study gave consistent results in three independent protoplast experiments.

DISCUSSION

3' terminal 90 bases of the polymerase gene are essential for viral replication

Mapping the sgRNA1 promoter of BYDV-PAV is not straightforward, because the sgRNA1 start site (2670) is within the 3' end of the essential polymerase gene. In this study, a premature stop codon introduced 90 bases from the 3' end of the polymerase gene abolished BYDV-PAV replication in protoplasts (Fig. 4). Considering this result, we decided to use a point mutagenesis approach rather than the deletion analysis to map the subgenomic promoter. An analogous premature stop codon mutation in the polymerase gene of BWYV was shown to be lethal for viral replication (Reutenauer *et al.*, 1993).

sgRNA1 transcription start site at base 2670

Mapping of luteoviral sgRNAs has proven to be a difficult process leading to conflicting reports. For example, Tacke *et al.* (1990) reported the 5' end of PLRV sgRNA to be at position -40 relative to the CP start codon, whereas Miller and Mayo (1991) mapped it to -212. Similarly, Dinesh-Kumar *et al.* (1992) mapped the sgRNA1 start site of BYDV-PAV-Illinois isolate to base 2769, whereas Kelly *et al.* (1994) mapped it to 2670 in BYDV-PAV-Australian isolate. To verify the correct start site of BYDV-PAV sgRNA1, we introduced mutations near base 2670. Mutant 2670M, in which the sgRNA1 start base (G at 2670) was altered to C, greatly reduced genomic and sgRNA1 accumulation. It is interesting how a single base or amino acid mutation can affect the viral replication. Reduction of sgRNA accumulation in response to alteration of putative

sgRNA transcription start site has been demonstrated for AlMV (van der Vossen *et al.*, 1995). Kel6 and Kelf mutants reduced sgRNA1 accumulation but did not greatly affect the genomic RNA accumulation (Fig. 5B). These two mutants indicate that the nucleotides immediately flanking the sgRNA1 start site are important for sgRNA1 activity. Similar obervation has been noted for CNV (Johnston and Rochon, 1995). Recent results (G. Koev, personal communication) from northern blot analysis using probes specific for start sites at 2670 and 2769 have also confirmed the result that the sgRNA1 start site is at base 2670.

With sgRNA1 start site at 2670, Kelly *et al.* (1994) have shown that the start sites of sgRNA1 and sgRNA2 occur at a sequence that closely resembles the 5' end sequence of the genomic RNA (5' AGUGAAGA). Identical sequences at the 5' termini of genomic and sgRNAs have also been reported for several other plant viruses, e.g., tobacco rattle virus (Cornelissen *et al.*, 1986), BMV (Marsh *et al.*, 1988), cowpea chlorotic mottle virus (Allison *et al.*, 1989), AlMV (van der Kuyl *et al.*, 1990), PLRV (Miller and Mayo, 1991), maize chlorotic mottle virus (Lommel *et al.*, 1991), CMV (Boccard and Baulcombe, 1993) and RCNMV (Zavriev et al., 1996). It has been proposed (Cornelissen *et al.*, 1986) that a direct repeat of the viral genomic 5' end at transcription start sites of sgRNAs represents a (-) strand signal for the viral replicase for (+) strand RNA synthesis. Another important conserved sequence, ACAAA is a candidate sgRNA promoter sequence in luteoviruses.

Role of ACAAA in virus replication

The complement of the conserved ACAAA sequence found at or near the 5' ends of genomic and sgRNAs of luteoviruses is postulated to be a putative origin of replication and sgRNA promoter sequence (Fig. 6; Miller *et al.*, 1995). The ACAAA sequence occurs many more times in most luteoviruses than would be expected by chance. 19 and 16 copies of ACAAA are found in PLRV and BYDV-PAV, respectively. Subgenomic leader sequences of a few luteoviruses are U-rich, resembling the sgRNA promoters of bromo viruses (Marsh *et al.*, 1988). The (-) strand sequence complementary to the ACAAA domain, perhaps in combination with the U-rich regions, may act as a promoter for replicase binding and initiation of RNA synthesis (Miller *et al.*, 1995). Even though three models have been proposed (Miller *et al.*, 1997) for sgRNA synthesis, there is no direct evidence on the mechanism of sgRNA synthesis for plant viruses, except for internal initiation mechanism in BMV (Miller *et al.*, 1985).

In this study, an ACAAA sequence (2729-2733) located in the 3' end of the polymerase gene, 59 nt downstream of the sgRNA1 start site (2670) was mutated to determine its role in viral replication. Mutating the ACAAA motif without any change in the amino acid sequence, reduced both the genomic RNA and sgRNA1 accumulation in oat protoplasts (Fig. 5B). RCNMV has an ACAAA sequence at the 5' ends of genomic RNA and sgRNA. Alteration of the ACAAA to ACUAA eliminated sgRNA synthesis of RCNMV (Zavriev *et al.*, 1996). Both the subgenomic promoter element and a putative stem-loop structure in minus-strand have been speculated to be essential for sgRNA

synthesis of RCNMV (Zavriev *et al.*, 1996). Recently, a conserved ACCA motif (*cis*-acting element) in the 5' nontranslated region of potato virus X has been shown to be essential for sgRNA synthesis (Kim and Hemenway, 1996).

Conserved sequences and secondary structure near the transcription start site of subgenomic RNA1

Sequences near the sgRNA1 start site were highly conserved in subgroup I luteoviruses (Fig. 7A). Computer-predicted secondary structure for several members of luteovirus subgroup I revealed an conserved stem-loop structure on the minus-strand near the sgRNA1 transcription start site (Fig. 7B). The secondary structure contained a seven base stem and a seven base loop with the sgRNA1 start site in the stem. A conserved ACUG sequence was always present in the loop. Recently, secondary structures in the putative sgRNA promoter regions have been reported for RCNMV (Zavriev *et al.*, 1996) and TCV (Wang and Simon, 1997). It is hypothesised that the stem-loop structure is essential for the recognition of polymerase and sgRNA synthesis (Zavriev *et al.*, 1996). The predicted secondary structure for sgRNA1 mutants (Kel6, Kelf and 2670M) are different from the stem-loop structure of subgroup I luteoviruses (compare Fig. 7C with 7B). According to predictions using MFOLD, Kel6 and 2670M mutants have a longer loop (10 bases) and Kelf mutant has significantly different secondary structure (Fig. 7C). Altered secondary structures might be the reason for lack of sgRNA1 accumulation in

these sgRNA1 mutants. Work is underway to identify the role of secondary structure in sgRNA1 synthesis.

Significance of subgenomic promoters

Mapping the subgenomic RNA promoters and understanding the biology of sgRNA synthesis is of great significance as the subgenomic promoters are putative origin of replication and putative hotspots for viral recombination (Miller *et al.*, 1995). Acting as an origin of replication, subgenomic promoter region of BMV inhibited viral replication when added in *trans in vivo* (Huntley and Hall, 1993). Similarly, an antisense RNA to the leader of sgRNA1 of BYDV-PAV inhibited viral replication in protoplasts (Mohan and Miller, unpublished data). In the future, we hope to exploit the subgenomic promoter sequences as replication origin to amplify antiviral and possibly other useful genes in transgenic plants. Currently, work is in progress to understand the mechanism of subgenomic RNA synthesis, to map the 5' and 3' borders of sgRNA1 promoter and to identify any *cis*-acting elements involved in sgRNA1 synthesis.

ACKNOWLEDGMENTS

The authors thank Dr. Chris Brown and Dr. Shanping Wang for technical advice and support with respect to site-directed mutagenesis, and Guennadi Koev for providing results prior to publication. This work was funded by USDA Biotechnology Risk

Assessment Grant. This is paper No. J- of the Iowa State University Agricultural and Home Economics Experiment Station.

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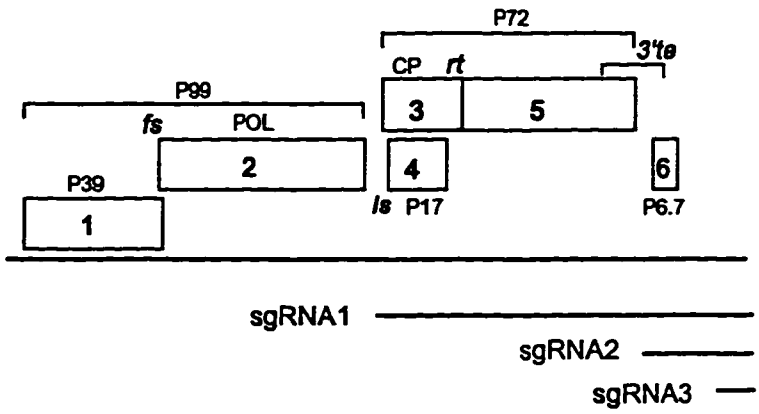
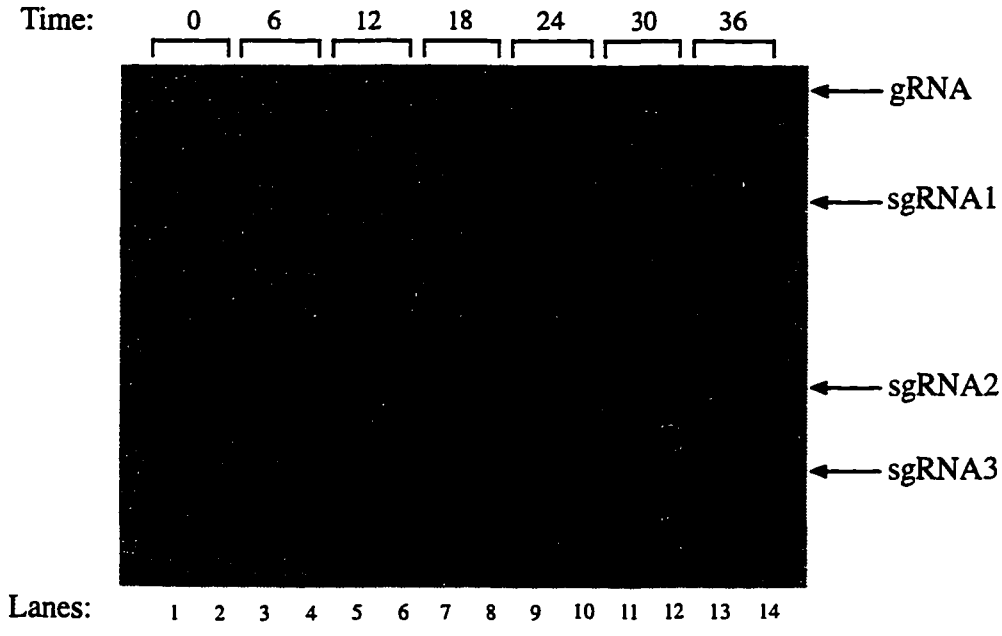


FIG. 1 Genome organization of BYDV-PAV. Open reading frames (ORFs) are indicated by number inside the box and by size of protein product outside the box. Sites of translational events are abbreviated as follows: fs = frameshift; ls = leaky scanning; rt = readthrough; 3'te = 3' translation enchancer. Bold lines below ORFs indicate genomic and subgenomic RNAs. POL indicates putative RNA-dependent RNA polymerase gene (ORF2). CP indicates coat protein gene (ORF3).

FIG. 2 BYDV-PAV genomic (g) and subgenomic (sg) RNA accumulation. **A** and **B**. Time course of the accumulation of BYDV-PAV genomic RNA and sgRNAs in oat protoplast. Protoplasts were inoculated with equal amounts of wild-type BYDV-PAV transcripts. Total RNA extracted at the times indicated (in hours postinoculation) over each lane were subjected to electrophoresis through denaturing agarose gels and analyzed by Northern blot hybridization. Blots were probed with transcripts from pSP10, complementary to the 3'-terminal 1450 bases of the PAV genome. Mobilities of the genomic RNA and sgRNAs are shown on the right.

A


Time:
0
6
12
18
24
30
36
gRNA
sgRNA1
sgRNA2
sgRNA3
Lanes:
1 2 3 4 5 6 7 8 9 10 11 12 13 14

B

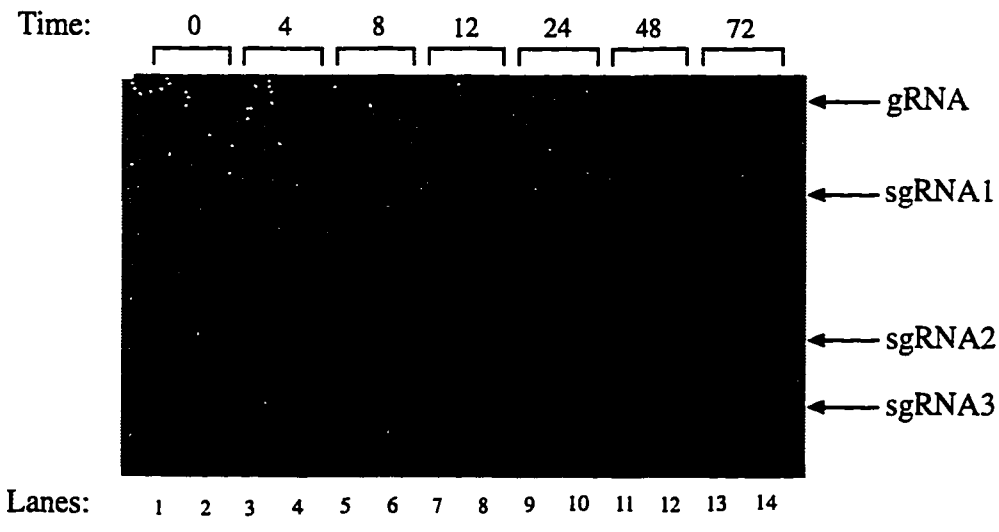
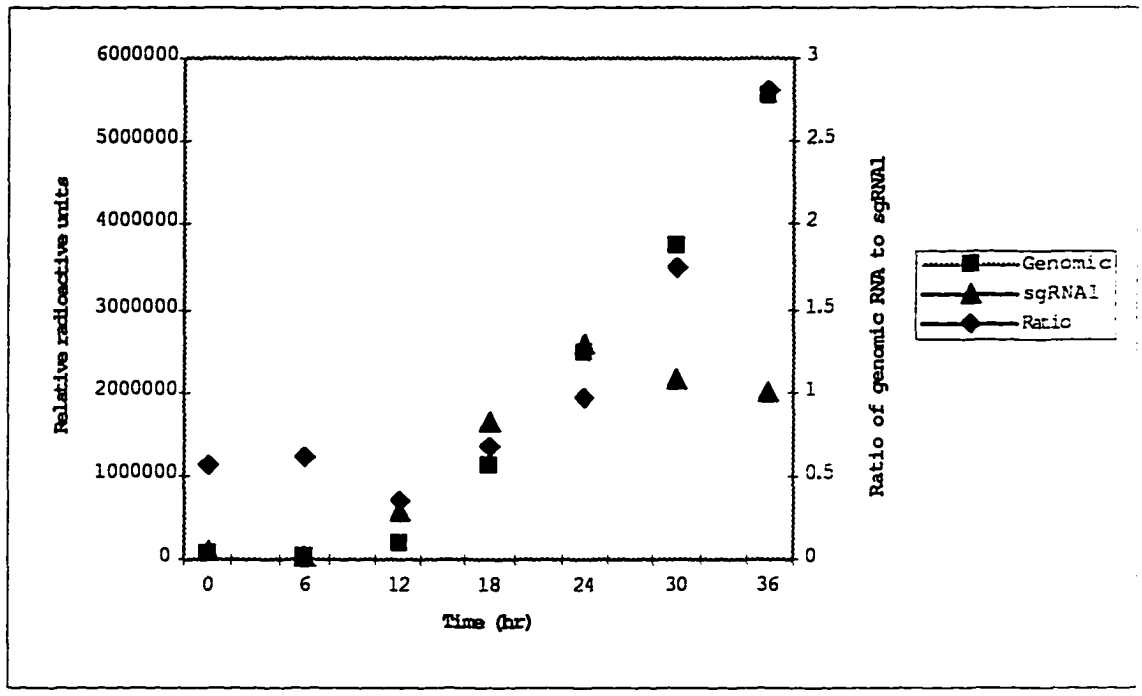

Time:
0
4
8
12
24
48
72
gRNA
sgRNA1
sgRNA2
sgRNA3
Lanes:
1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIG. 3 Time course of accumulation of BYDV-PAV genomic RNA and subgenomic RNA1 (sgRNA1) and ratios of genomic RNA to sgRNA1 in oat protoplasts quantified using Molecular Dynamics 840 phosphoimager and Imagequant 4.2 software from Northern blot hybridizations shown in Fig. 2. Abbreviations used: sgRNA1 = subgenomic RNA1; ratio = ratio of genomic RNA to sgRNA1. Ratios and time course accumulation from 0 to 36 hours (A) and 0 to 72 hours (B) postinoculation are shown.

A


6000000
5000000
4000000
3000000
2000000
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0
Relative radioactive units
3
2.5
2
1.5
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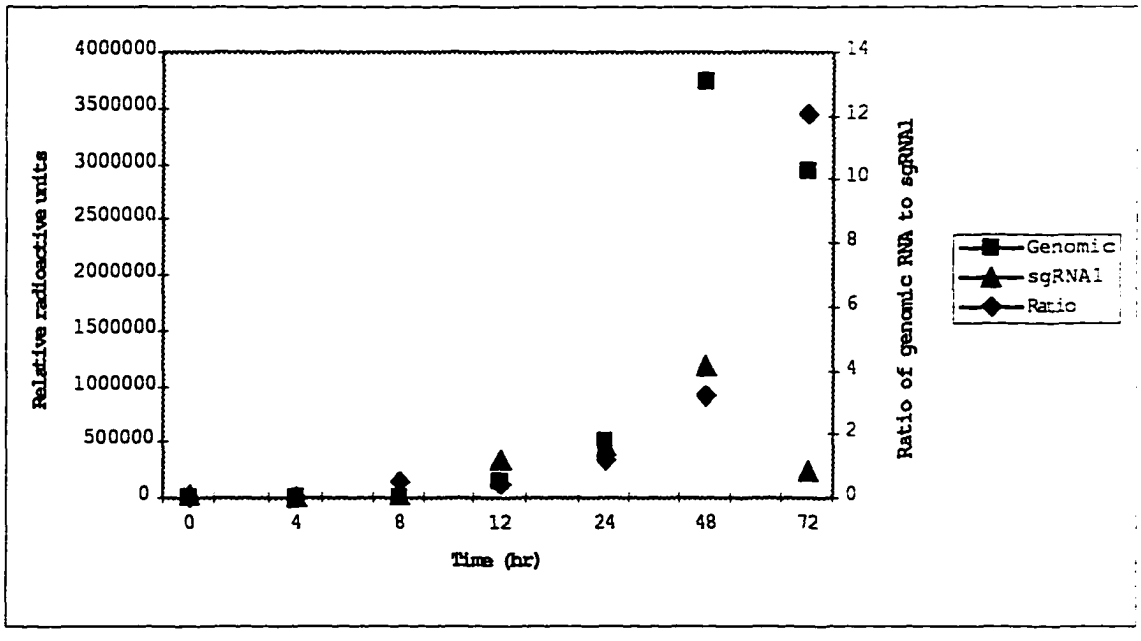
Ratio of genomic RNA to sgRNA1
0
6
12
18
24
30
36
Time (hr)
Genomic
sgRNA1
Ratio

B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2500000
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1000000
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0
Relative radioactive units
14
12
10
8
6
4
2
0
Ratio of genomic RNA to sgRNA1
0
4
8
12
24
48
72
Time (hr)
Genomic
sgRNA1
Ratio

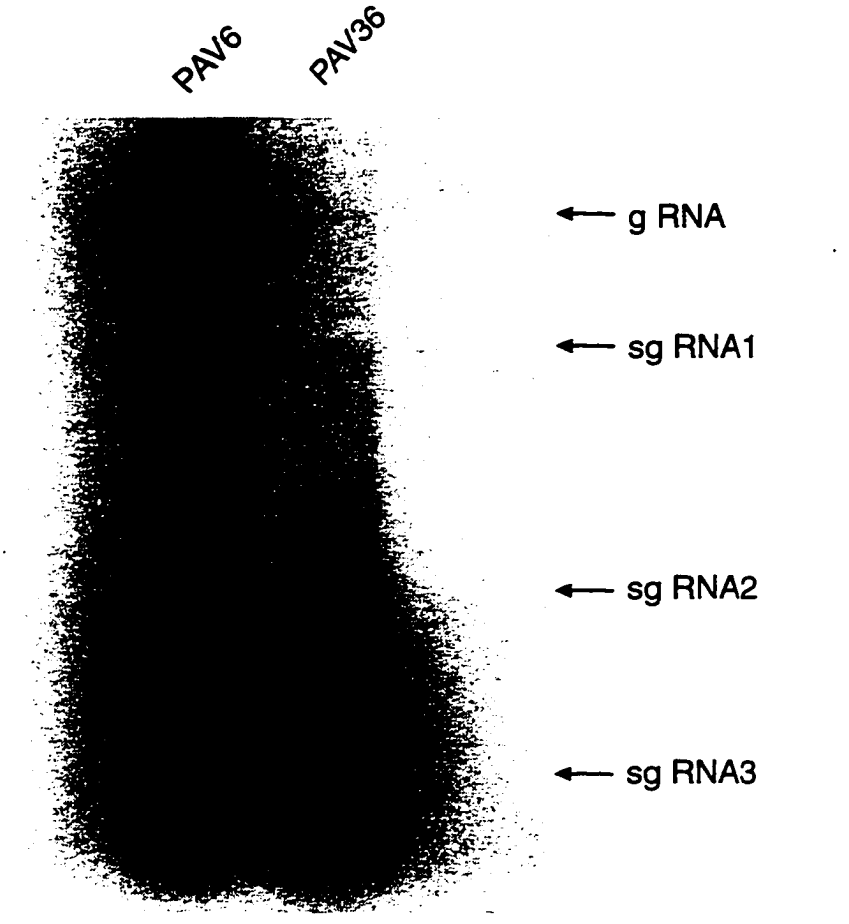


FIG. 4 Northern blot hybridization of RNA from cells inoculated with wild-type PAV6 and mutant PAV36 transcripts. Total RNA was extracted from protoplasts 48 hours after inoculation with the transcripts indicated above the lane. Blots were probed with transcript from pSP10, complementary to the 3'-terminal 1450 bases of BYDV-PAVgenome. Mobilities of genomic (g) and subgenomic (sg) RNAs are shown on the right.

FIG. 5 BYDV-PAV subgenomic RNA1 (sgRNA1) mutants. **A.** Full-length clones of BYDV-PAV genome containing the indicated mutations near the sgRNA1 start site. Mutated bases are bold italized. Arrows denote the transcription start site (base 2670) of sgRNA1. Transcription initiation base (G at 2670) of sgRNA1 and the conserved ACAAA sequence are shown in bold. **B.** Northern blot hybridization of RNA from cells inoculated with wild-type PAV6 and subgenomic RNA1 mutant transcripts. Total RNA was extracted from protolasts 48 hours after inoculation with the transcripts indicated above each lane. Blots were probed with transcript from pSP9, complementary to bases 2737-2985 of BYDV-PAV genome. Mobilities of genomic (g) RNA and subgenomic (sg) RNA1 are shown on the left.

A

PAV6 (wt): 2670
CAG AGU GUG AAG GUG
Ser Val Lys

1. KEL6:
CAG *UCG* *GAU* AAG GUG
Ser Asp Lys
5 bases and 1 amino acid (a .a) altered

2. KELf:
CAG *UCC* GU*U* AA*A* GUG
Ser Val Lys
5 bases altered without any change in a. a sequence

3. 2670M:
CAG AGU CUG AAG GUG
Ser Leu Lys
1 base and 1 amino acid altered

PAV6 (wt): 2729
CAC UCA CAA AAC
His Ser Gln Asn

4. ACAAA2:
CAC *AGC* CA*G* AAC
His Ser Gln Asn
4 bases altered without any change in a. a sequence

B

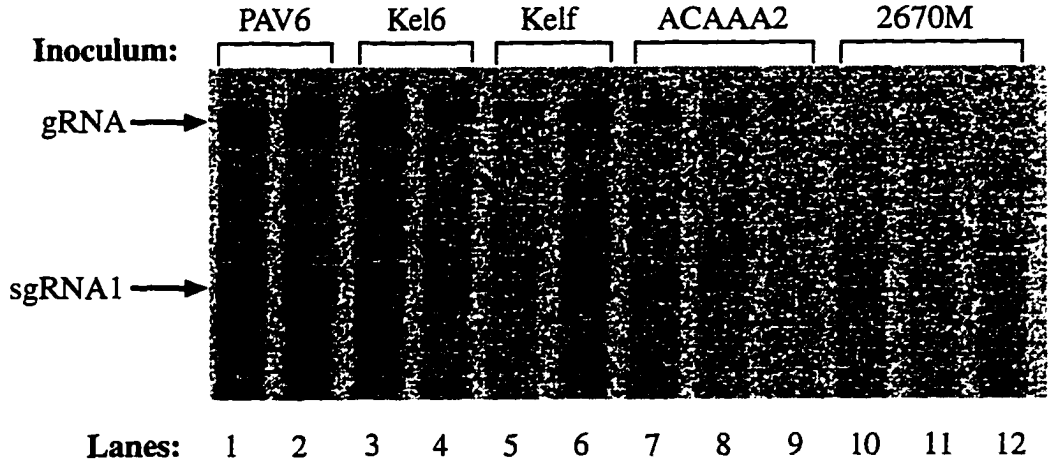


FIG. 6 Alignments of 5' ends and known and proposed (indicated by ?) subgenomic (SG) RNA transcriptional start sites of luteoviruses and selected related viruses (modified from Miller *et al.*, 1995). Nucleotide positions of the first base of each sequence is indicated by the preceding number. Bold letters indicate similarity between sequences. Known subgenomic start sites are in underlined italics. Dashes indicate gaps added to improve alignments. Abbreviations and references used are as in the text. **A.** Subgroup II luteoviruses and selected other related viruses. **B.** Subgroup I luteoviruses. The start site of BYDV-PAV sgRNA1 at base 2670 (Kelly *et al.*, 1994) is shown. A nine-base tract (CUCACAAAA) located 47 nt downstream of base 2670 is shown because it is identical to bases 17 to 25 near the 5' end of the genome.

A.

SBMV-C-5'	1	C**ACAAAA**UAUAAGA	Sobemoviruses
SBMV-C-SG?	3240	G**ACAAAA**CCGCGCG	
SBMV-B-SG?	3162	C**ACAAAA**UUAAUUU	
RYMV-5'	1	**ACAA**UUGAAGCUA	
RYMV-SG?	3440	C**ACAAA**GAUGGCCA	
PLRVA-5'	1	**ACAAAA**GAAUACC	Subgroup II luteoviruses
PLRVA-SG	3377	**A**CAAAAGAACACU	
BWYV-5'	1	**ACAAAA**GAA-ACC	
BWYV-SG	3259	**A**CAAAAGAU-ACC	
RPV-5'	1	**ACAAA**-GAUUACC	
RPV-SG?	3545	**ACAAAA**CUAAACU	
RPV-SG?	3562	**ACAAAA**CUAGCCG	
RPV-SG?	3575	G**ACAAAC**GUAAGUU	
RCNMV1-5'	1	G**ACAAAC**GUUUUAC	Dianthoviruses
RCNMV1-SG	2366	A**A**CAAAC**G**UAUUAC	
RCNMV2-5'	1	G**AAAC**CUCGCUC	
SCNMV1-SG?	2363	A**ACAAAC**GUAUUAC	
CRSV1-SG?	2297	A**ACAAAC**UUUUUAC	

B.

PAV-5'	1	**AGUGAAGA--UUGAC**CAU-----**CUCACAAAA**
PAV-SG1	2669	U**G**UGAAGG---**UGAC**GA.43nt.**CUCACAAAA**
PAV-SG2	4809	**A**GUGAAGACAACACCACUAGC**ACAAA**U
PAV-SG3	5329	**AUUGAAGA**CG**UU**AAAACUCG-**A**C**GA**CC
PAV-Jpn	1	**AGUGAAGA--UUGAC**CAU-----**CUCACAAAA**
PAV-Jpnsg1	2669	c**G**UGAAGG---**UGAC**GA.43nt.**CUCACAAAA**
SDV-5'	1	**AGUAAAG---UUGAC**ACCUUU**ACAGAA**
SDV-SG?	2731	U**GUAAAGAGAUUGAC**GCCUUU**ACUAGA**

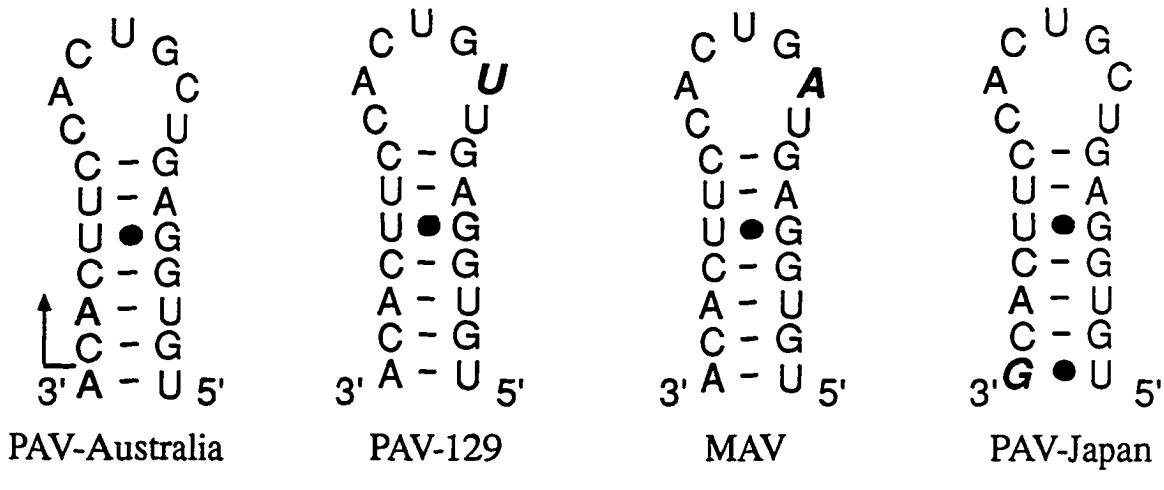
FIG. 7 Conserved sequences and secondary structure near the sgRNA1 transcription start site of subgroup I luteoviruses. **A.** Alignment of sequences near the sgRNA1 start site (base 2670) of subgroup I luteoviruses. Abbreviations used: AUS = Australia, PUR = Purdue. JPN = Japan. Conserved ACAAAA sequence and conserved 10 bases near sgRNA1 start site are boxed. Arrow indicates sgRNA1 transcription start site. **B.** Computer-predicted stem-loop structures presented with the minus-strand of MAV and PAV isolates in the region containing sgRNA1 start site. Arrow indicates start site of sgRNA1 transcription. **C.** Computer-generated secondary structures for sgRNA1 mutants. Minus-strand sequence is shown.

A

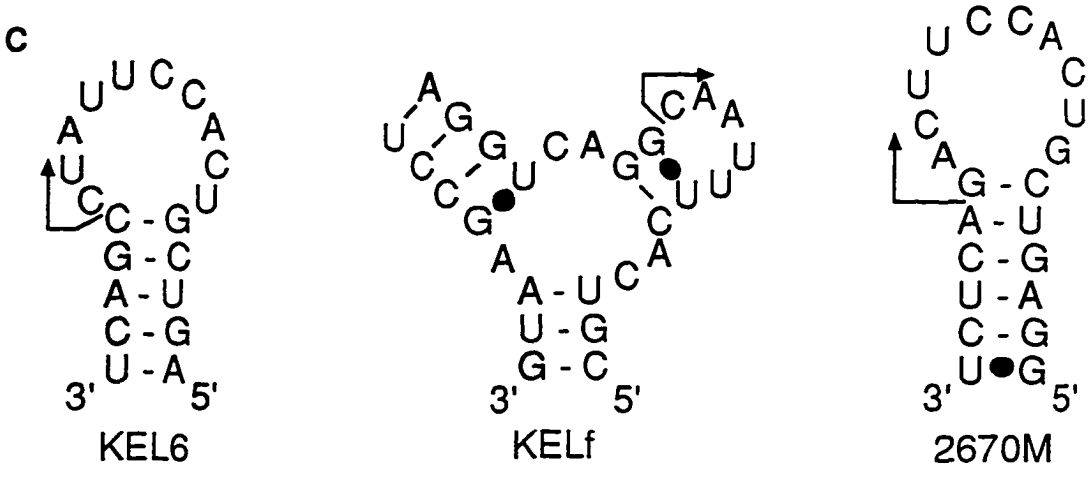
	2660			2699
PAV-AUS	ACUCCAGAGu	GUGAAGGUGA	CgACUCCACA	UCUGCAAUCA
PAV-PUR	ACUCCAGAGc	GUGAAGGUGA	CgACUCCACA	UCUGCAAUCA
PAV-JPN	ACUCCAGAGc	GUGAAGGUGA	CgACUCCACA	UCUGCAAUCA
PAV-129	ACUCCAaAGu	GUGAAGGUGA	CauCUCCACA	UCUGCAAUCA
MAV	ACUCCAGAGu	GUGAAGGUGA	CuACUCCACA	UCUGCAAUCA
Consensus	ACUCCAGAG-	GUGAAGGUGA	C-ACUCCACA	UCUGCAAUCA

	2700			2739
PAV-AUS	AUACUgCUUU	CCAUACCGGA	AAACCACUCA	CAAAACGAAU
PAV-PUR	AUACUaCUUU	CCAUACCGGA	AAACCACUCA	CAAAUCGAAU
PAV-JPN	AUACUaCUUU	CCAUACCGGA	AAACCACUCA	CAAAACGAAU
PAV-129	AUcCUcCUcU	CCAUuCCGGA	gAACaACUCg	CAAAACGAAU
MAV	AUACUaCUUU	CCAUACCGGA	AAACCACUCA	CAuAACGAAU
Consensus	AUACU-CUUU	CCAUACCGGA	AAACCACUCA	CAAAACGAAU

B



C



CHAPTER 5. GENERAL CONCLUSIONS

Barley yellow dwarf luteovirus (BYDV)-PAV RNA has a positive-sense, ~5.7kb RNA genome encoding at least six open reading frames (ORFs; Miller *et al.*, 1988a). The first two ORFs located at the 5' half of the BYDV-PAV are translated directly from genomic RNA (Di *et al.*, 1993). The 3' half of the genome is expressed from two subgenomic mRNAs (sgRNAs; Dinesh-Kumar *et al.*, 1992; Kelly *et al.*, 1994). BYDV and other luteoviruses use several strategies to express its genes. These strategies include the transcription of sgRNAs (Dinesh-Kumar *et al.*, 1992), readthrough of an amber termination stop codon (Tacke *et al.*, 1990), translational frameshifting (Brault and Miller, 1993). the expression of overlapping, out-of-frame ORFs by leaky scanning (Dinesh-Kumar and Miller, 1993) and cap-independent translation (Wang and Miller, 1995). The results of my research on BYDV replication are summarized below.

Genetically engineered resistance to BYDV

The ultimate goal of my research was to obtain transgenic oat plants resistant to BYDV infection. Antiviral strategies such as antisense (AS) RNA, sense (S) RNA, functional polymerase (POL) and modified polymerase (100% frameshift; 100%FS), were tested for their ability to inhibit viral replication in oat protoplasts. 3' sense RNA, POL, 100%FS and antisense to sgRNA1 leader (SG-AS) gave efficient inhibition (~80%) of BYDV virus accumulation in protoplasts, as monitored by enzyme-linked immunosorbent assay (ELISA) (Fig. 2 and 5; Chapter 2). However, none of the above constructs showed

any decrease in viral RNA accumulation in Northern blot analysis (Fig. 4; Chapter 2). Transgenic oat plants expressing SG-AS, POL and 100%FS were generated in collaboration with Dr. Somers (University of Minnesota). Currently, transgenic plants are being screened and tested for BYDV resistance.

Future research

Until recently, the progress on genetically engineered resistance to BYDVs has been relatively slow compared to other model plant viruses, due to the difficulty of transformation and regeneration of BYDV host plants. Although initial attempts of obtaining transgenic resistance to luteoviruses have not been very successful, efforts should continue to test new anti-viral strategies. Ribozyme-175 (see appendix 1), which has been shown to cleave the BYDV-PAV RNA *in vitro* (Silver, 1993), can be introduced into stably transformed oat plants to test its activity in plants. In addition, new anti-BYDV ribozymes should be designed and tested because of the great potential of ribozymes in suppressing replication of RNA in plants (Yang *et al.*, 1997). Several new antisense RNA and sense RNA constructs, targeting different regions of the BYDV-PAV genome, should be constructed. It will be interesting to test an antisense RNA construct targeted to the 3'-translation enchancer (3'-TE) element because 3'-TE has been shown to stimulate translation of uncapped BYDV-PAV genomic RNA (Wang and Miller, 1995). All these anti-viral constructs should be first tested in the transient oat protoplast system, before

moving on to the more time and labor consuming task of transforming and regenerating transgenic oat plants.

Transgenic plants expressing the defective replicase genes have conferred resistance to many plant viruses (Anderson *et al.*, 1992; Longstaff *et al.*, 1993; Brederode *et al.*, 1995). Mutated or truncated replicase genes of BYDV-PAV should be constructed and tested for transgenic resistance. Mutating the characteristic GDD motif or introducing a deletion in the replicase gene can create a defective replicase gene. Trangenic plants expressing only ORF1 or ORF2 of BYDV-PAV can be tried for transgenic BYDV resistance.

Replication of BYDV

Determining the viral genes and sequences needed for translation and replication of BYDV is essential for designing efficient antiviral strategies. Deletion and mutation analyses were performed to identify gene products and *cis*-acting signals involved in replication and encapsidation of BYDV-PAV RNA. Any deletion in ORFs 1 and 2 destroyed viral infectivity. Analogous ORFs of BWYV were shown to be essential for viral replication (Reutenauer *et al.*, 1993). *In vitro* translation of the deletion mutants revealed that the sequence upstream of the shifty heptanucleotide was required for ribosomal frameshifting, and that a 3'-translational enhancer stimulated translation more efficiently when placed in closer proximity to the translated ORFs. Deletion of the coat protein gene reduced genomic RNA accumulation. The carboxy-terminally extended form

of the coat protein gene, produced by translational readthrough of its stop codon . was not required for replication or encapsidation. Although the ORF6 gene product was not necessary, *cis*-acting RNA signals in and around ORF6 were essential for viral replication. BYDV-PAV RNA replication may be coupled to translation, because defective RNAs containing various deletions were not replicated in *trans* by the co-inoculated wild-type helper genome (Mohan *et al.*, 1995).

Future research

The results of my research have identified the genes and *cis*-acting sequences involved in translation and replication of BYDV-PAV (Mohan *et al.*, 1995). These results are significant because they lead to conclusions different from those of Young *et al.* (1991). Small new deletions in different parts of the BYDV-PAV genome can be constructed . Attempts should be made to replicate these new defective RNAs in presence of wild-type helper BYDV-PAV transcripts in *trans*-complementation experiments. Absence of *trans*-complementation led Mohan *et al.* (1995) to suggest *cis*-preferential replication for BYDV-PAV. However, the possibility of *trans*-replication cannot be ruled out because certain combinations of new defective RNAs may be able to overcome the *cis*-limitation. More studies have to be done on ORF6 to identify its role in BYDV-PAV replication because the 3'-half of ORF6 is the most variable portion of the 3'-region among 10 geographically different PAV-like isolates of BYDV (Chaloub *et al.*, 1994)

Necessity of ORF6 for viral replication can be determined by introducing small deletions and mutations in ORF6 of BYDV-PAV.

Mapping the subgenomic mRNA promoter

Subgenomic promoters are putative hotspots for viral recombination and putative origin of replication (Miller et al., 1995). The transcription start site of subgenomic RNA1 (sgRNA1) was identified to be at base 2670 by site-directed mutagenesis. Time course accumulation of the sgRNAs paralelled the accumulation of genomic RNA. Mutating the transcription initiation base, G at 2670, or the nucleotides immediately flanking it, reduced sgRNA1 accumulation in protoplasts. Computer-predicted secondary structures in the putative sgRNA1 promoter region of many members of subgroup I luteovirus revealed a conserved stem-loop structure near the sgRNA1 transcription start site. A conserved ACAAA sequence has been found at the 5' ends of genomic and sgRNAs of many luteoviruses (Miller *et al.*, 1995). Mutating this ACAAA domain reduced both genomic RNA and sgRNA1 accumulation. The 3' terminal 90 bases was also found to be essential for BYDV-PAV replication in oat protoplasts.

Recommendations for future research

Bases in and around the sgRNA1 transcription start site are expected to be a part of the sgRNA1 promoter. Although the sgRNA1 start site has been identified to be at base 2670 (Kelly *et al.*, 1994; Mohan and Miller, chapter 4), the exact 5' and 3'-borders of the

sgRNA1 promoter has not been mapped yet. Deletion analysis cannot be used to map the sgRNA1 promoter because the sgRNA1 start site is well within the polymerase gene, which has been shown to be essential for BYDV-PAV replication (Mohan *et al.*, 1995). Thus, PCR-based techniques can be used to delineate the boundaries of sgRNA1 promoter. An alternate approach is to insert the fragment containing the putative sgRNA1 promoter in the middle of ORF5, which is not required for BYDV-PAV replication (Filichkin *et al.*, 1994; Mohan *et al.*, 1995). If the fragment contains an active sgRNA promoter, then a new additional sgRNA should accumulate in the infected protoplasts or plants. Such ectopic expression of sgRNAs to map the sgRNA prmoters has been reported for AIMV (van der Vossen *et al.*, 1995) and TCV (Wang and Simon, 1997). The putative sgRNA1 promoter region inserted in ORF5 can now be subjected to deletion analysis to map the sgRNA1 promoter. It will be interesting to map the sgRNA2 promoter of BYDV-PAV because the putative sgRNA2 promoter region might include the 3'-translation enhancer element.

After mapping sgRNA1 and sgRNA2 promoters of BYDV-PAV, their core promoter sequences can be compared with the putative subgenomic promoters of other plant viruses. Computer-aided sequence alignments can identify any conserved motif among the sgRNA promoter sequences. Using these sequence alignment studies, a consensus subgenomic promoter sequence may be obtained for plant viruses in general andd luteoviruses in specific. In the minus-strand of many members of subgroup I luteovirus, a conserved computer-predicted stem-loop structure has been identified near

the sgRNA1 start site (Mohan and Miller, chapter 4; G. Koev, personal communication). The necessity of this proposed stem-loop structure for sgRNA1 accumulation can be tested by introducing compensating mutations that weakens the proposed secondary structure.

Mutating the conserved ACAAA motif (bases 2729-2733) reduced both genomic RNA and sgRNA1 accumulation (Mohan and Miller, chapter 4). Another ACAAA sequence, located in the 5'-region (bases 19-23) of BYDV-PAV, should be mutated to determine the necessity of ACAAA motif for genomic RNA and sgRNA accumulation. Mapping the subgenomic promoters, identifying the *cis* and *trans*-acting elements required for the accumulation of genomic RNA and subgenomic RNAs should lead us to our ultimate goal of understanding the viral replication better and designing efficient anti-viral genes to BYDV-PAV.

APPENDIX 1. TRANSIENT EXPRESSION OF RIBOZYME AS AN ANTIVIRAL AGENT TO BARLEY YELLOW DWARF LUTEOVIRUS IN OAT PROTOPLASTS

Ribozymes are catalytic RNA molecules that possess enzymatic, self-cleavage activities (for reviews see Symons, 1991; Edgington, 1992). Naturally occurring ribozymes are derived from certain plant satellite RNAs and viriods (Uhlenbeck, 1987; Haseloff and Gerlach, 1988). Several features of ribozymes make them attractive as potential antiviral agents, in particular their specificity of cleavage and ability to cleave multiple substrate molecules.

Guidelines for design of synthetic hammerhead ribozymes have been reported by Haseloff and Gerlach (1988). The ribozyme consists of a catalytic hammerhead domain flanked by two hybridizing 'arms', which are complementary to the target RNA. The length of the arms can vary from 6 nucleotides to much longer antisense sequences. The antisense sequences facilitate binding of the ribozyme to its target and correct positioning of the catalytic domain with respect to cleavable motif in the target RNA. In most naturally occurring hammerhead ribozymes, cleavage occurs 3' of a GUC trinucleotide. Synthetic ribozymes can also catalyse efficient *in vitro* cleavage adjacent to GUA, GUU, CUC and UUC trinucleotides.

A number of examples have been described where the ribozyme approach has been used for gene suppression *in vivo*. (Cameron and Jennings, 1989; Cotten and Birnstiel,

1989; Sarver *et al.*, 1990; Scanlon *et al.*, 1991). In contrast to the numerous reports of ribozyme activity in animal and human cells, there are only a few reports in plant cells. Reduction of neomycin phosphotransferase (NPT) activity by ribozymes was achieved in plant protoplasts (Steinecke *et al.*, 1992). Wegener *et al.* (1994) expressed ribozymes in transgenic tobacco to reduce NPT activity. De Feyter *et al.* (1996) demonstrated that a ribozyme gene and an antisense gene are equally effective in confering resistance to TMV in transgenic tobacco. Recently, Yang *et al.* (1997) reported ribozyme-mediated high resistance against potato spindle tuber viroid in transgenic potatoes

Anti-BYDV ribozymes

Miller *et al.* (1991) showed that the satellite RNA of BYDV contains a novel hammerhead structure in the self-cleavage domain. Later, several ribozymes were designed in our laboratory (Silver, 1993) to cleave the genomic RNA of BYDV-PAV. Of the three ribozymes that were tested, ribozyme-175 (Rz-175) was shown to cleave most efficiently *in vitro* (Silver, 1993). Rz-175 was targeted to the 5'-end of ORF1 of BYDV-PAV because cleavage in the 5'-end of the virus may inhibit an early step in virus replication. To test the ribozyme activity *in vivo*, a reporter gene, β-glucuronidase (GUS) assay was employed.

Plasmids used and results of GUS assays

The 5'-end of BYDV-PAV genome was fused to the GUS reporter gene so that expression of GUS required translation of the 5'-end of ORF1 including the Rz-175 target site. Cleavage of the fused ORF1-GUS mRNA would thus prevent translation of GUS. Plasmids were constructed such that this gene was transcribed from either the cauliflower mosaic 35S or maize Adh1 promoters. Plasmids pAGUS1 and pAdhGUS have GUS gene in front of 35S and Adh1 promoter, respectively. Plasmids p35S.RzSub and pAdh.RzSub have ribozyme substrate (RzSub; 5' 528 bases of BYDV-PAV) and GUS gene fused to 35S and Adh1 promoter, respectively. Plasmids p35S.Rz and pAdh.Rz encoded Rz-175 behind 35S and Adh1 promoter respectively. As a negative control, plasmids p35S.ΔRz and pAdh. ΔRz were constructed (ΔRz = defective ribozyme). These two differed from Rz-175 by a single A to C base change in the required CUGA sequence. This allows base-pairing between ribozyme and target RNA but eliminates cleavage activity and thus permits us to differentiate between the true cleavage and antisense-mediated inhibition due to ribozyme base-pairing to the target. The ribozyme-encoding and GUS-encoding plasmids were co-electroporated into oat protoplasts. After 24 hours of incubation, protoplasts were harvested and GUS activity was measured by GUS assay, as described previously (Dinesh-Kumar and Miller, 1993).

The results of the two independent GUS assays are shown in Table 1. In experiment one, plasmids encoding both Rz-175 and defective Rz-175 inhibited GUS activity by about 50% (Table 1; compare 502 and 512 with 1200 for Adh constructs and

2031 and 2056 with 4423 for 35S plasmids). In experiment two, plasmids encoding both Rz-175 and ΔRz-175 gave GUS readings similar to that of ribozyme substrate alone (Table 1; compare 1200 and 1153 with 1250 for Adh plasmids). In two other experiments, the GUS values of Rz-175 and ΔRz-175 were not significantly different from that of ribozyme substrate alone (data not shown). These results indicate that plasmids encoding Rz-175 and ΔRz-175 might not inhibit GUS gene activity. Similar results were obtained by Mazzolini *et al.* (1992), who reported that the high level expression of ribozymes in transiently transformed protoplasts fails to inhibit target gene expression.

To test the ribozyme activity *in vivo* by a different assay, we used ELISA to monitor virus accumulation. RNA transcripts of Rz-175 and ΔRz-175 were co-electroporated with BYDV-PAV RNA into oat protoplasts. After 24 hours of incubation, protoplasts were harvested and ELISA was performed using BYDV-PAV-specific antibody. ELISA values (A_{405}) of BYDV-PAV + Rz-175 (1.08) and BYDV-PAV + ΔRz-175 (1.01) were not significantly different from that of BYDV-PAV alone (A_{405} = 1.27). Similar results were obtained in another protoplast experiment (data not shown). These ELISA results indicate that Rz-175 and ΔRz-175 does not inhibit BYDV-PAV virus accumulation in protoplasts. The exact reason(s) for the inability of the ribozymes to cleave the BYDV-PAV RNA are not clearly known. Stability of the small ribozyme molecules *in vivo* and the length of the flanking sequences which influences the binding and dissociation of ribozyme are two key factors for the success of ribozymes *in vivo*.

Table 1. *In vivo* activity of ribozyme against BYDV-PAV as monitored by GUS assay

Plasmids[a]	Average GUS readings[b]	
	Experiment 1	Experiment 2
pAdh.RzSub	1200	1250
pAdh.RzSub + pAdh.Rz	502	1200
pAdh.RzSub + pAdh.ΔRz	514	1153
p35S.RzSub	4423	
p35S.RzSub + p35S.Rz	2031	
p35S.RzSub + p35S.ΔRz	2056	
Salmon sperm DNA	25	22
pAdh.GUS		6430
pAdh.GUS + pAdh.Rz		6001

[a]Abbreviations used: Rz = ribozyme; RzSub = ribozyme substrate and ΔRz = defective ribozyme

[b]GUS readings are average of two replications.

Although initial attempts of obtaining an efficient anti-BYDV ribozyme have been unsuccessful, efforts should continue to design and test ribozymes because of their enormous potential in gene suppression. Ribozymes should be tested in stably transformed plants because in protoplasts, the antiviral and viral RNA arrive at the same time but in transgenic plants, the constitutively expressed antiviral gene would already be present at higher levels than the invading viral RNA. Anti-BYDV ribozymes could be successful if they are properly designed and expressed

APPENDIX 2. SYNERGISTIC INTERACTIONS BETWEEN SUBGROUP I AND SUBGROUP II LUTEOVIRUSES

Higher plants commonly experience infection with several different viruses at a time, and a number of plant diseases are attributed to a synergistic interaction between two unrelated viruses in the same plant (Barker *et al.*, 1987; Calvert and Ghabrial, 1983). Mixed infections of two viruses produce disease symptoms much more severe than those caused by either of the viruses alone. Accumulation of many luteoviruses is enhanced in mixed infections with other luteoviruses or their relatives.

Mixed infection of BWYV and BWYV ST9-assosiated RNA (ST9aRNA) greatly exacerbates disease symptoms (Falk and Duffus, 1984). ST9aRNA is like an umbravirus, which lacks CP but are capable of autonomous replication. Umbraviruses have a luteovirus subgroup I-like polymerase gene and depend on subgroup II luteoviruses for aphid transmission. Carrot motley dwarf disease is caused by a mixed infection of carrot mottle umbravirus (CMotV) and carrot red leaf luteovirus (CRLV; Table 1). CRLV acts as a helper virus for aphid transmission of CMotV (Waterhouse and Murant, 1983).

Groundnut rosette disease results from a synergistic infection of groundnut rosette umbravirus (GRV) and groundnut rosette assistor luteovirus (GRAV; Table 1). GRAV helps in virus transmission of GRV (Murant *et al.*, 1988). Pea enation mosaic enamovirus has two RNAs, each of which can replicate independently in plant cells, but depend on each other for movement and encapsidation functions (Demler *et al.*, 1993). All of these

Table 1. Synergistically interacting pairs of viruses and RNAs

Polymerase homology			
Subgroup II (-like)[a]	Subgroup I (-like)[a]	Host	Effect
BYDV-RPV	BYDV-PAV	Cereals	Severe stunting
BWYV	ST9a RNA	Beet, Lettuce, Shepherd's purse	Severe stunting
CRLV	CMoV (umbra)	Carrot	Carrot motley dwarf disease
GRAV	GRV (umbra)	Groundnut	Groundnut rosette disease
PEMV RNA1 (enamo)	PEMV RNA2 (enamo)	Legumes	Ability to infect plants

[a]Virus group assigned to the above non-luteovirus or RNAs are in parentheses. Abbreviations used are as in the text.

viruses and RNA have been found in various pairs in which an RNA coding for a subgroup I luteovirus-like polymerase enhances replication of an RNA coding for a subgroup II luteovirus-like polymerase (Table 1; modified from Miller *et al.*, 1997).

BYDV-PAV and BYDV-RPV synergy

Considering the above examples for synergism from lueoviruses, synergistic interaction between BYDV-PAV and BYDV-RPV was investigated at the protoplast level. Previously, our laboratory and others (P. M. Waterhouse, Australia) have observed that mixed infection of BYDV-PAV and BYDV-RPV gave higher virus yield and more

severe disease symptoms than infection by either virus alone . Oat protoplasts were electroporated with transcripts of BYDV-PAV alone, BYDV-RPV alone and mixture of BYDV-PAV and BYDV-RPV RNA. After 48 hours, virion accumulation was assayed by ELISA.

BYDV-PAV alone and BYDV-PAV + BYDV-RPV gave average ELISA values (A_{405}) of 0.71 and 0.92, respectively with a PAV-specific antibody. ELISA value with RPV-specific antibody of BYDV-PAV + BYDV-RPV (0.73) was not significantly different from that of BYDV-RPV alone (0.54). Thus, no higher ELISA values were observed for BYDV-PAV + BYDV-RPV. Recently, Koev (1996) reported that the oat protoplasts coinfected with BYDV-PAV and BYDV-RPV RNAs showed no higher levels of RNA accumulation than when inoculated with either viral RNA alone. However, plants infected with both BYDV-PAV and BYDV-RPV showed more severe disease symptoms than infection by either virus alone (Koev, 1996). These results indicate that the effect of disease severity in mixed infections might not be due to increased levels of RNA or virion accumulation of either BYDV-PAV or BYDV-RPV.

The exact mechanism(s) of viral synergism are unknown. Vance *et al.* (1995) have demonstrated that PVX interacts synergistically with potato virus Y potyvirus (PVY) and three other members of the potyvirus group. Unlike BYDV-PAV and BYDV-RPV synergism, PVX-PVY synergism caused a change in the regulation of PVX RNA replication and an increase in PVX accumulation (Vance *et al.*, 1995). The region of the potyviral genome that conferred synergy did not include the polymerase gene. In contrast,

the one feature common to the luteoviral synergisms is the paired polymerases of divergent origin (Table 1). Thus, the most likely candidate for the luteoviral synergism is the polymerase gene (Miller *et al.*, 1997). The mechanism of synergism and the protein, RNA sequences and genes involved in synergistic interactions needs to be determined.

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